Identification of MDM2-interacting proteins in renal cell carcinoma and characterization of phenotypic properties acquired by renal cell carcinoma cells which over-express MDM2 and p53

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By

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Abstract

High levels of MDM2 are associated with poor prognosis in renal cell carcinoma (RCC) patients. This effect does not seem to be a result of MDM2-mediated degradation of p53, as those cancers which display high levels of MDM2 usually also express high levels of p53. Over-expression of p53 and MDM2 is associated with decreased disease free survival in patients with RCC. Since most patients who die as a consequence of RCC do so as a result of metastatic spread, we therefore hypothesised that MDM2 may contribute to cellular processes that lead to increased metastatic potential of RCC tumours. In addition, we further hypothesised that MDM2 might accomplish this through promoting some cellular mechanisms, such as motility and invasiveness, which could contribute to metastasis in RCC patients.

In this study we have found that increased expression levels of the MDM2 protein do drive increased motility and invasiveness of RCC cells. Additionally, we have demonstrated that MDM2 expression is driven by p53 in RCC cell lines expressing wt p53. Moreover, using FASAY and DNA sequence analysis, we have shown that upregulated p53 in clear cell RCC tumour samples is generally wild type which suggests that the high levels of MDM2 in these tumours are likely to be promoted by p53. Therefore, our results suggest that p53 indirectly induces increased motility of RCC cells, at least in part, through contributing to over-expression of MDM2.

Nearly all of the known functions of MDM2 are mediated through protein-protein interactions. Therefore, one aim additional of this project was the identification of new proteins that are targeted by MDM2 in RCC cells in order to try to explain how MDM2 may contribute to poor outcome in RCC patients. We performed a yeast two hybrid screen using MDM2 as the bait to identify such MDM2 interacting proteins. In order to maximise the likelihood of identification of interactions that may indeed

provide clues for elucidation of an aggressive phenotype of RCC, an RCC cell line over-expressing p53 and MDM2 (therefore reflecting the situation observed in tumours) was used as a source of mRNA for library construction. Several (novel) putative MDM2-interacting proteins were detected and one of these: NME2 was selected for further analysis for two major reasons. Firstly, NME2 was independently identified as an MDM2 interacting protein in our laboratory using a proteomic approach and secondly NME2 was previously shown to function as a suppressor of motility and metastasis. Using isogenic cell lines expressing either relatively high or low levels of MDM2, it was shown that the motility suppressive function of NME2 was compromised in RCC cells expressing high levels of MDM2. Our results suggest that MDM2 may act to promote motility by opposing or directly blocking NME2-dependent motility suppression.

Several additional novel functions of NME2 in the regulation of MDM2 and p53 were also revealed. NME2 was shown to negatively regulate the activity of p53 in the MDM2-independent manner. Moreover, NME2 was demonstrated to reduce the steady-state level of MDM2 and alter the MDM2-dependent post-translational modifications of p53, thus showing that NME2 may play a pleiotropic role in regulation of the p53 and MDM2 network.

Altogether the present study identifies a novel function of MDM2 in promoting motility and invasiveness of RCC cells and provides other clues to understanding of the processes which potentially contribute to an aggressive phenotype of renal cell carcinoma.

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Podziękowania

Z całego serca pragnę podziękować moim najdroższym i najwspanialszym rodzicom oraz mojej najukochańszej Ulce za miłość, cierpliwość, wsparcie na które zawsze mogłem liczyć i zapewnienie mi poczucia bliskosci, które nie opuszczało mnie przez cały czas pracy nad tym przedsięwzięciem.

Dziękuję Kochani!

Declaration of originality

Hereby I declare that the present thesis was composed by myself and was not presented in any previous examination for a degree. All sources of information as well as several figures in the Introduction section, that were either adapted or reproduced, are acknowledged by means of references. All experimental work presented in the Results Section was performed by me, except for the experiment shown in Figure 3.11b, which was performed by Dr Maria Maguire.

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List of abbreviations

Abbreviation	Definition
AD	activation domain
Amp	ampicillin
β2AR	β2-adenergic receptor
ATR	ATM-related polypeptide
APAF1	apoptotic protease activating factor 1
APC	adenomatus polyposis coli gene
ATM	ataxia- telangiectasia mutated
Cdk	cyclin dependent kinase
Cul2	cullin 2
DBD	DNA binding domain
DNA-PK	DNA-dependent protein kinase
DO media	drop-out media (do not contain certain nutrients, thus provide selection for cells carrying selectable marker genes)
dsDNA	double stranded DNA
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FASAY	functional analysis of separated allele in yeast
FDA	food and drug administration
FBS	Fetal Bovine Serum
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GSK3	glycogen synthase kinase 3

HACCH	hexaminocobalt chloride
HIF-1	hypoxia inducible factor 1
IR	ionizing radiation
IARC	International Agency for Research on Cancer
JNK	C-Jun Amino-terminal kinase
Kan	kanamycin
LD-PCR	long distance PCR
LMP1	latent membrane protein 1
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
MEFs	mouse embryonic fibroblasts
NER	nucleotide excision repair
NICE	National Institute for Health and Clinical Excellence
NME	proteins from the nucleoside diphosphate kinase (NDPK) family, also called NM23,
O/N	over night
PCAF	p300/CBP-associated factor
PDGF	platelet derived growth factor
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PIs	protease inhibitors
PTEN	phosphatase and tensin homologue deleted on chromosome ten
Rbx1	RING-box 1
RCC	renal cell carcinoma
RLU	relative light units
RT	room temperature

SOS	son of sevenless
SSCP	single-strand conformation polymorphism
ssDNA	single stranded DNA
STAT	signal transducers and activators of transcription
STI	soybean trypsin inhibitor
TERT	telomerase reverse transcriptase
TF	transcription factor
TRRAP	transformation/transcription domain-associated protein
TSG	tumour suppressor genes
VHL	von Hippel-Lindau
VEGF	vascular endothelial growth factor
Wnt	wingless, a morphogen identified in drosophila, responsible for limb morphogenesis
Wt	wild type

1 Introduction

1.1 Overview

Over-expression of p53 and MDM2 correlates with poor outcome in RCC (renal cell carcinoma) patients (Haitel et al., 2000). However, over-expression of p53 alone is not associated with such a highly aggressive phenotype of RCC tumours. It was therefore speculated that MDM2, rather than p53, is responsible for tumour progression resulting in the poorest outcome. However, the exact mechanism by which MDM2 functions to accomplish this in RCC remains unknown. Based on the clinical evidence presented by Haitel et al., two major research strategies were applied in order to elucidate the mechanism/s by which renal tumours over-expressing MDM2 become more aggressive. The first strategy aimed at the identification of novel MDM2 interacting proteins in RCC followed by analysis of phenotypic characteristics of RCC cells in which levels of these molecules were manipulated. The second strategy involved an investigation of the p53-MDM2 relationship in RCC and in RCC cells. Since over-expression of MDM2 has been reported to correlate with up-regulation of p53 (Haitel et al., 2000, Moch et al., 1997), it could be suggested that over-expression of MDM2 may somehow depend on upregulation of p53.

In order to familiarize the reader with the principles of this project, several issues related to RCC, such as association of over-expression of p53 and MDM2 with poor patient outcome will be presented in following sections. This will be followed by a review of the literature describing the functions and regulation of p53 and MDM2, with particular emphasis on the relationship between p53 and MDM2 as this is likely to be malfunctioning in RCC.

1.2 Renal cell carcinoma – introduction

1.2.1 Incidence and environmental risk factors of RCC

Kidney cancer is diagnosed in around 6,700 people each year in the UK, which is approximately 2% (3% in men and < 2% in women) of all diagnosed cancer cases. Every year, over 3,600 patients die as a result of this disease and this number is increasing. There are roughly 200,000 new cases of kidney cancer diagnosed worldwide each year, with various frequencies within different populations ranging from around 15 per 100,000 population in Czech Republic to around 1 per 100,000 population in India (Cancer Research UK statistics, 2002). Similarly to the UK, the incidence of kidney cancer worldwide is rising (Patel et al., 2006). Although several factors, such as: cigarette smoking, obesity, hypertension, hormonal therapy as well as heavy metal ions, asbestos and petroleum products, are known to increase risk of developing kidney cancer, it is not clear which (if any) of these contribute and to what extent to the increasing incidence (Motzer et al., 1996).

1.2.2 Classification of renal tumours

RCC (renal cell carcinoma) is the most frequent type of kidney cancer and accounts for 85% of all cases (Cancer Research UK statistics, 2002). The most common types of RCCs as well as their most characteristic features are summarised in the Table 1.1. As the Table shows, there are a plethora of RCC sub-types. However, only the clear cell, papillary or chromophobe types occur in 5% (or more) of cases. Out of these three, the clear cell subtype is not only the most common, but appears also to be the most aggressive one (Lopez-Beltran et al., 2009). Additionally, sarcomatoid RCC (a histological variation characterised by the presence of tumour cells with morphology

resembling various types of mesenchymal cells) may arise from any type of RCC (Delahunt, 1999).

RCC subtype	Incidence	Prognosis	Genetic	Cell/tissue characteristics
Clear cell	75%	Aggressiveness according to grade, stage and sarcomatoid change	–3p, +5q22, –6q, –8p, –9p, –14q	Clear cytoplasm; cells with eosinophilic cytoplasm occasionally)
Multilocular cystic	Rare	No progression or metastases	VHL gene mutation	Clear cytoplasm, small dark nuclei
Papillary	10%	Aggressiveness according to grade, stage and sarcomatoid change	+3q, +7, +8, +12, +16, +17, +20, -Y	Type 1 (basophilic) or type 2 (eosinophilic)
Chromophobe	5%	10% mortality	–1, –2, –6, –10, –17, –21, hypodiploidy	Pale or eosinophilic granular cytoplasm
Collecting ducts of Bellini	1%	Aggressive, 2/3 of patients die within 2 years	–1q, –6p, –8p, –13q, –21q, –3p (rare)	Eosinophilic cytoplasm
Medulary	Rare	Mean survival of 15 weeks after diagnosis	Rare loss of chromosome 22	Eosinophilic cytoplasm
Xp11 translocation	Rare	Indolent	t(X;1)(p11.2;q21), t(X;17)(p11.2;q25), Other	Clear and eosinophilic cells
After neuroblastoma	Rare	Related to grade and stage	Allelic imbalance at 20q13	Eosinophilic cells with oncocytoid features
Mucinous tubular and spindle cell	Rare	Rare metastases,	–1, –4, –6, –8, –13, –14 +7, +11, +16, +17	Tubules, extracellular mucin and spindle cells
Unclassified	4% to 6%	High mortality	Unknown	Variable, sarcomatoid
Renal cell neoplasms in end-stage renal disease	Rare	Unknown	Variable gains chromosomes 7 and 17	Clear cells or eosinophilic cells
Thyroid follicular carcinoma-like tumor of kidney	Rare	Unknown	Gains 7q36, 8q24, 12, 16, 17p11-q11, 17q24, 19q, 20q13, 21q22.3, and Xp. Losses of 1p36, 3, 9q21–3	Clear cells
Tubulocystic carcinoma	Rare	Unknown	Variable trisomy of chromosome 17	Eosinophilic cells

Table 1.1 **Types of RCC**. The Table summarises selected features characteristic to the most common types of RCC. Reproduced from (Lopez-Beltran et al., 2009).

There are several criteria according to which RCC tumours can be classified. The TNM staging system is based on the TNM (Tumour Node Metastasis) classification and is presented in Table 1.2. TNM staging is the most commonly used system for classification of tumours and prediction of the outcome in patients (Ficarra et al., 2007). Based on this system, the five-year disease specific survival for stages I, II, III and IV has been reported as 89.6%, 82.7%, 57.7%, and 18.3%, respectively (Elmore et al., 2003). In addition to the TNM classification, other systems are used to classify RCC tumours and evaluate prognosis.

T – primary tumour			
T0	there is no evidence of a primary tumour in the kidney		
T1	the tumour is no more than 7cm across and is completely inside the kidney		
T2	the tumour is more than 7cm across, but is still completely inside the kidney		
T3	the cancer has spread through the kidney capsule, to a major vein, the adrenal gland or other tissues immediately surrounding the kidney		
T4	the cancer has spread further than the tissues immediately surrounding the kidney		
N – regional lymph node			
N0	No cancer in any lymph nodes		
N1	Cancer spread to one nearby lymph node only		
N2	Cancer spread to more than one nearby lymph node		
M – distant metastasis			
M0	Absence of distant metastasis		
M1	Presence of distant metastasis		
Staging criteria			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1,T2	N1	M0
	T3	N0, N1	M0
Stage IV	T4	N0, N1	M0
	Any T	N2	M0
	Any T	Any N	M1

Table 1.2 **Tumour stages based on the TNM (Tumour Node Metastasis) classification** (Cancer Research UK, classification of RCC tumours).

For example, the Fuhrman nuclear grading system, which is based on evaluation of the shape of nuclei (see Table 1.3), is widely used for classification of renal tumours. This classification scheme has been shown to be a valuable tool for prediction of metastatic disease (Fuhrman et al., 1982).

Grade	Characteristics of nuclei
1	Small, regular, homogeneous, nucleoli invisible
2	Larger than in grade 1, variable size, occasionally irregular, nucleoli visible at high microscopic power
3	Large, irregular, with large nucleoli,
4	Large, very irregular and multi-lobed, with very distinct nucleoli and chromatin clumps

Table 1.3 **Fuhrman's nuclear grading system** (Fuhrman et al., 1982).

1.2.3 Management of RCC

RCC is well known for its unpredictability as the aggressiveness of similarly staged or graded tumours using conventional histological techniques, even within the same type of RCC (e.g. clear cell RCC), appears to be highly variable (Rouviere et al., 2006, Beisland 2004). Additionally, RCC tends to be asymptomatic; however, if the symptoms are present (typical symptoms associated with RCC include hematuria, flank mass and flank pain), they often occur late in the disease course and are rarely present together. In many cases the disease remains undetected until it reaches an advanced stage. In fact, 40-60% of diagnoses are made incidentally in developed countries, when patients undergo abdominal CT scanning for other complaints (Jayson, 1998). Additionally, various kinds of treatment, such as radiotherapy and conventional chemotherapy, are ineffective, especially in case of metastatic disease (Motzer et al., 1996, NICE Technology Appraisal Guidance 178). Therefore, the lack of reliable disease course prediction markers, late detection and resistance to conventional therapies, contribute to the RCC mortality rate and make the disease very difficult to manage. Surgery is a basic treatment for RCC which often cures early stage kidney cancers (20-30% relapse rate in stages 1 and 2) (NICE Guidance

on Cancer Services). Nephron sparing (for small tumours) and radical nephrectomy (for more advanced disease) is a standard treatment not only for patients with localised disease; prolonged survival has also been observed in patients with metastatic disease undergoing radical nephrectomy (Motzer et al., 1996). Surgery is also performed as a part of palliative treatment and is usually offered to all patients who are fit enough to survive the operation (NICE Guidance on Cancer Services).

Postoperative treatment for advanced RCC has historically included IL-2 and IFN-α, but the overall response rate to these drugs is only around 15% (Reeves and Liu, 2009). IL-2-based therapy has been shown to result in complete remissions, though, at a low rate and accompanied by severe side effects restricting the number of patients eligible for this treatment (Reeves and Liu, 2009). Additionally, these therapies have also been shown to be ineffective for treatment of the early-stage tumours (NICE Guidance on Cancer Services).

Several new therapeutic strategies to treat advanced RCC are based on a new generation of drugs. These include Sunitinib, an inhibitor of RTK (receptor tyrosine kinase); Bevacizumab, a monoclonal antibody which inhibits VEGF (vascular endothelial growth factor); or Sorafenib, which inhibits Raf kinase function. Additionally, drugs targeting the mTOR pathway were recently introduced to the clinic. mTOR inhibitors, such as Temsirolimus in 2007, significantly increase the overall survival, progression free survival and the objective response rate (measurable response such as changes in tumour size) in patients with metastatic disease (Rini, 2008). The drugs mentioned above have recently been approved NICE (NICE Technology Appraisal Guidance 178). However, clinical effectiveness as a first-line treatment for patients with advanced and/or metastatic RCC (Bevacizumab, Sorafenib and Temsirolimus) and second-line treatment for patients with advanced

and/or metastatic RCC (Sorafenib and Sunitinib) is limited (Motzer et al., 2006, Yang et al., 2003, FDA, NICE Technology Appraisal Guidance 178). The overall response to these drugs measured according to RECIST (Response Evaluation Criteria in Solid Tumours, see Table 1.4) (Padhani and Ollivier, 2001), reaches up to 40% and extend the progression free survival to up to 11 months compared with IFN-α alone (median time to progression to 5.4 months). However, among the targeted therapies for advanced RCC (specific inhibitors or monoclonal antibodies) presented above, only Temsirolimus and Sunitinib appeared to increase overall survival compared to IFN-α. Application of targeted therapies often results in high initial response; however, the tumours eventually adapt and become resistant (Patel et al., 2006). Therefore, there is a need for methods allowing early detection and therapies which will cure RCC rather than just delay disease progression. Various approaches, such as proteomic methods, are used to search for disease-specific targets for new therapies as well as disease prognostic markers. Two-dimensional gel electrophoresis combined with mass spectrometry has been applied to the identification of proteins aberrantly expressed in tumours; whereas antibody arrays have been used to identify tumour associated antigens (Craven and Banks, 2008). Identified proteins could be used as biomarkers or, potentially serve as therapeutic targets. Alternatively, high throughput methods such as DNA microarray analysis, not only allow measurement of expression of single genes, but also analysis of patterns of gene expression specific for the type and character of a disease. Recognition of these specific patterns could have prognostic value and could help to define which signalling pathways are altered in aggressive RCC. This would help to predict the disease course, and aid in planning of therapeutic strategies (Tan et al., 2004).

Type of response	**RECIST change in sum longest diameter**
Complete response (CR)	Disappearance of all target lesions; confirmed at 4 weeks
Partial response (PR)	At least 30% reduction in the sum of the longest diameter of target lesions, taking as reference the baseline study; confirmed at 4 weeks
Stable disease (SD)	Neither PR nor PD criteria are met, taking as reference the smallest sum of the longest diameter recorded since treatment started
Progressive disease (PD)	At least 20% increase in the sum of the longest diameter of target lesions, taking as reference the smallest sum longest diameter recorded since treatment started or appearance of new lesions

Table 1.4 Definition of response according to RECIST criteria. Adapted from (Padhani and Ollivier, 2001).

1.3 Molecular biology of renal cell carcinoma

As mentioned previously, RCC is a heterogeneous disease due to a large number of tumour types (see Table 1.1). This diversity is also manifested at a molecular level; there are a number of factors known to be associated with the pathogenesis of RCC. As Table 1 shows, the number of chromosomal aberrations typically observed in RCC is large and distinct for particular subtypes; certain abnormalities appear to be tumour-type specific, such as 3p loss in the clear cell subtype. One explanation for loss of the chromosome 3p in clear cell carcinomas is presence of the *VHL* gene (see the next section) on the short arm of the chromosome 3 (3p25.3) and therefore loss of heterozygosity of this tumour suppressor. The role of VHL in tumour suppression is described in the following section.

1.3.1 The VHL tumour suppressor in renal cell carcinoma

VHL (von Hippel-Lindau protein) is thought to be a critically important tumour suppressor in the kidney. Germline mutations in the *VHL* gene cause von Hippel-Lindau disease, often leading to development of tumours of which RCC, haemangioblastoma and pheochromocytoma are the most typical (Duan et al., 1995). The importance of the role of VHL in tumour suppression is further supported by the observation that this protein is mutated in over 50% of sporadic clear cell RCC cases, whereas hypermethylation of the *VHL* promoter has been found in a further 10-20% of cases (Gnarra et al., 1994). Mutations of *VHL* have also been observed in haemangioblastomas and pheochromocytomas (Kim and Kaelin, 2004). The tumour suppressor function of VHL is dependent on its ability to regulate the level of HIF-1 (hypoxia-inducible factor-1), a transcription factor that up-regulates a range of genes involved in angiogenesis; thus preventing a key step in tumour formation. VHL is composed of two functional domains, α and β (Iliopoulos et al., 1998). The α domain is responsible for organisation of the E3 ubiquitin ligase complex called ECV (Elongin-Cullin-VHL), which is essential for mediating the tumour suppressor function of VHL. This domain binds to elongin C, which interacts with CUL2 (cullin-2). CUL2, in turn, recruits RBX1 (RING-box 1), a protein mediating interaction with the E2 ubiquitin-conjugating enzyme. The β domain of VHL is responsible for recognition and interaction with a target protein to which ubiquitin is transferred by the ECV complex (Ohh, 2006). The ECV acts as an ubiquitin ligase for HIF1α. The process of ubiquitylation of HIF1α is regulated by presence of oxygen in the cellular environment. In the presence of oxygen, the proline residues 402 and 564 of HIF1α are hydroxylated, allowing for ECV-dependent ubiquitylation of HIF1α. This leads to its subsequent degradation by 26S proteasomes. Under

hypoxic conditions, however, the proline residues are not hydroxylated; as a result HIF1α is not degraded and may function to promote angiogenesis (Ohh, 2006).

Mutations of *VHL* result in loss of tumour suppressor function and, as previously mentioned, are common in RCC, particularly the clear cell sub-type (Duan et al., 1995). Inherited mutation in one allele of the VHL gene and loss of the second allele during life is typical of patients with von Hippel-Lindau disease. Loss of the second allele of a TSG is a phenomenon frequently observed in cancers, called LOH (loss of heterozygosity) (Singh et al., 2001). Penetrance of von Hippel-Lindau disease reaches 97% by the age of 60 with a median survival of 49 years (Maher et al., 1990). Although RCC is not the most common type of tumour in VHL patients (occurring in only 28%), it is the major cause of death in VHL disease. Interestingly, the most common VHL disease-associated tumours are heamangioblastomas which occur in around 65% of cases.

It has been suggested that the *VHL* mutation in human renal epithelial cells may be an early event in the evolution of RCC (Linehan et al., 1995). As shown in Figure 1.1, loss of *VHL* heterozygosity seems to trigger cytological changes. Depending on the type of *VHL* mutation (as several types of *VHL* mutations are known, see table 1.5), the renal epithelial cells begin to form cysts and over-express HIF1α.

Further genetic changes result in the formation of carcinomas (Kim and Kaelin, 2004). It appears, that VHL inactivation may be a critical event in the development of RCC, as restitution of wt *VHL* in RCC cell lines decreases their tumourigenic potential (Iliopoulos et al., 1995). However, it is important to establish what other genetic alterations may be responsible for carcinoma formation and for promoting the further progression into aggressive metastatic disease. Interestingly, analysis of mice with heterozygous *Vhl* knockout ($Vhl^{+/-}$) that were generated in order to mimic

the von Hippel-Lindau disease, revealed some rather unexpected results. The *Vhl*$^{+/-}$ animals did not develop tumours characteristic of VHL disease, suggesting a human-specific mechanism of RCC development associated with loss of VHL function (Kleymenova et al., 2004).

Recent studies have revealed new functions of VHL. The VHL protein was found to interact with p53 and protect it from MDM2 mediated inhibition and degradation (Roe et al., 2006). Moreover, VHL also seems to have the ability to increase transactivation activity of p53 by promoting acetylation of the carboxy-terminal residues K373 and K382 of p53 through stabilisation of a complex containing p300, pCAF and p53. This suggests that VHL has more anti-tumour activities than just degradation of HIF1α, and its loss may have a wide spectrum of effects contributing to tumourigenesis.

VHL mutation	HIF binding	Risk for RCC
Type I	None	High
Type IIA	Moderate	Low
Type IIB	Weak	High
Type IIC	Strong	None

Table 1.5 **Types of *VHL* gene mutations.** The table shows types of mutations of the *VHL* gene (left column) together with their effect on HIF binding (right column) and risk for RCC associated with this mutation. Type I mutations – deletions, nonsense and missense mutations resulting in a total loss of function. Type IIA, B and C – usually point mutations which modulate the ability of VHL to bind to HIF1α. Adapted from (Iliopoulos, 2006).

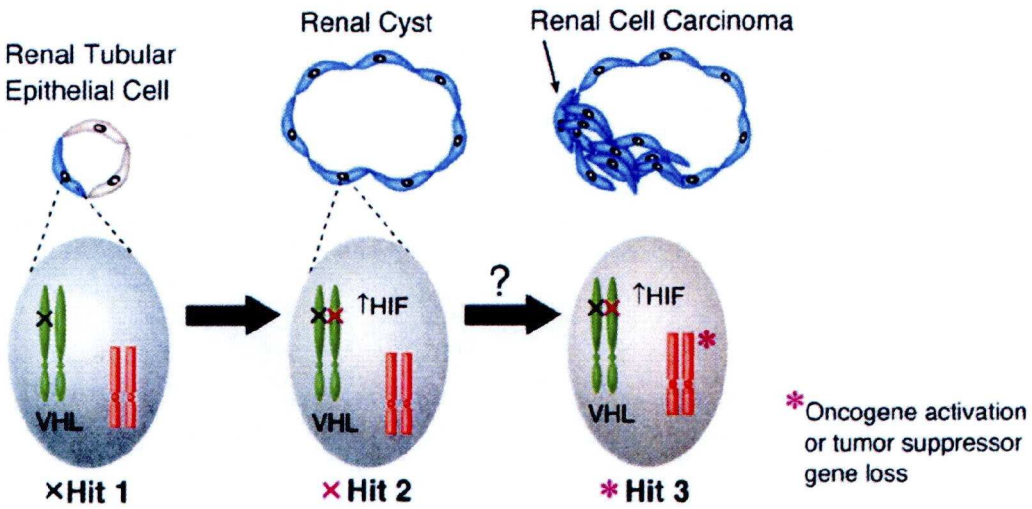


Figure 1.1 **The 3-hit model of RCC progression.** The first two mutational hits lead to loss of VHL function, the third involves inactivation of another TSG or activation of an oncogene. The second and third hits are associated with changes in behaviour of affected cells. Reproduced from (Kim and Kaelin, 2004).

1.3.2 Biomarkers implicated in RCC

Since loss of VHL activity/function occurs in the majority of RCC cases (Gnarra et al., 1994), analysis of VHL as a prognostic marker does not have any significant value. Therefore considerable effort has been focused on the identification of novel, more informative biomarkers.

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), markers of systemic inflammation have also been shown to have a relevant prognostic value in RCC. Elevated ESR has been shown to be associated with decreased disease specific survival ($p=0.0086$) and shorter progression free survival ($p=0.002$) in a study of 110 Japanese patients with a localised CCRCC (Sengupta et al., 2006).

CRP has also been shown to be an adverse indicator of survival (Lamb et al., 2006). The study has shown that patients with the levels of CRP ($n=45$) higher than 6mg/l had substantially reduced survival following the surgery than patients with the levels of CRP ($n=26$) lower than 6mg/l ($p=0.0046$).

Carbonic anhydrase IX (CAIX), an enzyme produced by renal cells, has been shown to be a predictor of poor outcome (Bui et al., 2003); reduced expression of CAIX is associated with markedly decreased survival of RCC patients.

Although several biomarkers such as these mentioned above have been identified, none of them are appropriate for diagnosis and evaluation of treatment outcome in RCC reviewed by Tunuguntla et al. Since, as mentioned before, the standard classification schemes often fail to predict the disease course, there is a need for more definitive biomarkers that will allow more accurate prognostication and thus enable stratification of patient treatment.

1.4 p53 and MDM2 in RCC

As mentioned above, renal cell carcinoma is an unpredictable disease, as neither the tumour stage nor the nuclear grading system and currently available biomarkers have proven to be sufficiently informative in many RCC cases. Therefore, there is a need for new ways of predicting tumour behaviour.

It has been observed that p53 is often over-expressed in high-grade renal tumours and there are a number of studies that investigated expression and mutational status of p53 in RCC. Several factors, however, constitute a source of variation between these studies. For instance, differences in tumour grade, stage and proportions of sub-types investigated in particular studies make it difficult to compare the data from different papers (Munro et al., 2005). Additionally, several different fixation techniques and antibodies were used for detection of p53 in these studies (reviewed in Noon et al., 2010 and shown in Table 1.6). It has been suggested, that the DO-7 antibody is more sensitive that the antibody 240 (Olumi et al., 2001). This might lead to generation of different results in studies using different reagents. However, despite these differences, it has been demonstrated, that p53 is over-expressed in about a quarter of all RCC tumours, including also other histological subtypes, such as papillary renal cell carcinoma (reviewed in Noon et al., 2010). It has been calculated that p53 is more often over-expressed in metastatic (51.8%) *vs* non-metastatic (22%) cancers (Zigeuner et al., 2004). Moreover, the majority of studies (presented in the Table 1.6) reported correlation of p53 over-expression with decreased survival (reviewed in Noon et al., 2010 and presented in Table 1.6).

Mutational analyses have also been performed in several studies; SSCP (single-strand conformation polymorphism) was usually used for screening of samples to detect mutated alleles which was followed by direct sequencing. The frequencies of

mutations appear to vary from 0 to 79% and as is the case in other cancers, missense mutations are the most common (Gad et al., 2007, Oda et al., 1995). However, the majority of studies reported the mutation frequency below 10% which is substantially lower than in other carcinomas such as colorectal, breast or lung. This might be due to heterogeneity of tumour sub-types among different studies and difficulty in detection of mutations in tumours rich in stromal components (reviewed in Noon et al., 2010). Since the screening process using SSCP is very laborious, the majority of studies investigated the sequence of exons 5-8 or 4-8 which encode the DNA-binding domain of p53. Although the majority of mutations (around 85%) occur within the DNA-binding domain of p53, significant number (around 15%) of mutations are found also outside of exons 5-8 (Hollstein et al., 1991). Therefore, the real number of mutations may be higher than reported in the studies reviewed by Noon et al.

As MDM2 over-expression is well known to be associated with poor outcome in various types of tumours such as: breast, gastric, non- Hodgkin's and Hodgkin's lymphoma and leukeamias (Yang et al., 2006, Ohmiya et al., 2006, Maestro et al., 1995, Chilosi et al., 1994, Lu et al., 2002, Bueso-Ramos et al., 1993), expression of MDM2 was also investigated in RCC. A study of 97 CCRCC specimens by Haitel et al. showed that over-expression of MDM2, present in 19% of cases, was associated with high tumour grade ($p=0.0149$) and decreased disease free survival ($p=0.00113$) (Haitel et al., 2000). The study also evaluated expression of p53, which appeared to be upregulated in 36% of tumour samples and correlated with decreased disease-free survival ($p=0.00291$). Interestingly, the study also showed that a subset of tumours co-over-expressed MDM2 and p53 and this was associated with the worst prognosis (disease free survival shorter than 1 year). In cases where only p53 was over-

expressed, this figure increased to around two years, whereas patients with normal p53 and MDM2 levels were likely to live more than five years without progression of the disease (p=0.00179).

Study	**No. of Specimens (% CCRCC),**	**Antibody**	**p53 prognostic value**	**Comments**
Klatte 2009	170(100), AS	NP	Yes	Decreased DFS
Perret 2008	50(0) all PRCC	DO-7	Yes	Decreased OS In type II PRCC
Phuoc 2007	119(100), AS	DO-7	Yes	Decreased DSS
Kankuri 2000	117(80), AS	DO-7	Yes	Decreased OS in patients with metastases
Kramer 2005	117(89), AS	DO-7	Yes	
Langner 2005	95(75), stage pT1	DO-7	No	
Cho 2005	92(100), AS	NP	Not evaluated	Decreased DSS
Shvarts 2005	193(85), AS	DO-7	Yes	20% cutoff predicted recurrance
Uzunlar 2005	57(77,1), AS	NP	Yes	Decreased DSS
Zigeuner 2004	184(70.7), AS, 56(94.8)[a]	DO-7	Yes	Decreased MFS in CCRCC
Kim 2004	318, AS	DO-7	Yes	Decreased DSS
Uchida 2002	112(78), AS	DO-7	Yes	
Olumi 2001	43(100), AS	DO-7/ PAB240/ both	No	Decreased DSS
Ljungberg 2002	99(74), AS	DO-7	Yes	Decreased survival in non-CCRCC
Girgin 2001	50(62)	DO-1	Yes	Decreased DSD
Haitel 2000	97(100)	DO-1	Yes	Decreased DSS
Rioux 2000	66(?), AS	DO-7	Yes	Decreased DSS
Sejima 1999	53(?), AS	RSP53	No	
Vasavada 1998	38(71), T1 and T2	DO-7	No	
Silik 1997	39(100), AS	DO-7	No	
Papadopoulos 1997	90(?), T1 and T2	DO-1	No	
Zhang 1997	70(?), AS	Ab-6	Not evaluated	
Gelb 1997	52(100), T1 and T2	DO-7	No	
Shiina 1997	72(?), AS	DO-7	Yes	Decreased OS
Moch 1997	50(100), T3	DO-7	Yes	Decreased OS
Hofmockel 1996	31(?), T1-T3	DO-7	No	
Chemeris 1995	82(40)	DO-1	Not evaluated	
Lipponen 1994	123(?)	CM1	Yes	Increased RFS
Kamel 1994	56(?), AS	CM1	No	
Bot 1994	100(74), T1-T3	DO-7	No	
Uhlmen 1994	175(?), AS	NP	Yes	Decreased DSS

Table 1.6 **Prognostic significance of p53 over-expression in RCC tumours.** The Table shows the results of studies investigating the prognostic significance of p53

over-expression in RCC tumours. Note that the Table also shows the percentage of clear cell RCC (CCRCC) analysed *vs* other RCC sub-types and antibodies used for IHC analysis. DFS, disease-free survival; OS, overall survival; DSS, disease-specific survival; MFS, metastasis-free survival; DSD, disease-specific death; RFS, recurrence-free survival; AS, all stages; NP, information not provided; [a]in addition to 184 renal cancers, 56 surgically removed metastatic tissues were analyzed. Adapted from Noon et al.

This suggests that over-expression of MDM2 (in addition to p53) may further increase the aggressiveness of RCC. Haitel et al. also show that p53/MDM2 double positivity is associated with presence of distant metastasis (p=0.003) and high tumour grade (grade 3 and 4, p=0.007) which suggest that p53 and MDM2 may serve as indicators of tumour progression. Intriguingly, high levels of MDM2 appeared to be highly associated with the presence of high levels of p53 (p=0.00004). This observation was also made by another study (p=0.0006) (Moch et al., 1997) and suggested that over-expression of p53 and MDM2 may be somehow connected (Noon et al., 2010).

Very little is known about the biological properties of RCC cells over-expressing p53 and MDM2. As will be discussed later (see Chapter 4), co-over-expression of p53 and MDM2 is not a common situation; it is not observed in normal cells or in most types of cancer cells (other than RCC). Therefore, several questions arise:

- Is MDM2 involved in tumour progression-promoting mechanisms in RCC? As cancer patients usually die due to expansion of distant metastasis (not due to growth of the primary tumour), it is not unreasonable to suggest that MDM2

could play a role in promoting this process, however, the exact (putative) role of MDM2 in RCC progression remains unknown.

- How does MDM2 become upregulated in RCC and what role does p53 play in this upregulation? As high levels of MDM2 in RCC are highly associated with the presence of high levels of p53, it could be suggested that expression of MDM2 may somehow depend on p53. However, as it is unclear if p53 is usually wt or mutated in high grade RCC tumours, it is not obvious if over-expression of MDM2 is due to increased p53-dependent transcription (reviewed in Noon et al., 2010). An alternative possible explanation is provided by studies of SNP at position 309 (SNP309) in the MDM2 promoter (a more detailed description of SNP309 will be presented in the next section). It has been observed, that the G/G variant of the 309 polymorphic site in the promoter of MDM2 is responsible for increased transcription of MDM2 in RCC tumours (Hirata et al., 2007). However, this study did not provide any information regarding survival and therefore whether the SNP309 haplotype is associated with altered survival is a question that has to be addressed in future studies.
- Why is p53 elevated in some RCCs and why is MDM2 not degrading p53 in these tumours? Several mechanisms leading to stabilisation of p53 exist. These are usually parts of stress responsive pathways (reviewed in Vousden, 2000). As there are a number of stresses present in cancer cells (such as DNA damage, oncogene activation), the possibility exists that these mechanisms also lead to p53 stabilisation in RCC. Alternatively, the ability of MDM2 to degrade p53 could be inhibited by some yet unknown mechanisms (reviewed in Noon et al., 2010).

As the key questions asked above remain generally unanswered, there is a need for a better understanding of the molecular biology of RCC tumours over-expressing p53 and MDM2. Analysis of the p53-MDM2 pathway, which is clearly altered in these tumours, may provide clues leading to the identification of components of the pathway that are not functioning normally in RCC.

The following part of the Introduction will therefore focus on the p53-MDM2 network, in order to highlight basic aspects of the normal regulation and pathophysiological deregulation of this complicated system.

1.5 The p53 tumour suppressor

1.5.1 Introduction to the p53 network

p53 was first identified as a transformation-related protein in (SV40) transformed cells *in vitro*, as well as in cancer cells. Since its expression was initially detected only in tumour tissues, it was concluded that *p53* might be an oncogene (DeLeo et al., 1979, Lane and Crawford, 1979, Linzer and Levine, 1979). This view was further supported by the observation that high levels of p53 correlated with increased cell proliferation in culture (Dippold et al., 1981). However, it has subsequently been demonstrated that the p53 studied in cancer cells, was in fact mutated and that the wild type form of p53 did not have any transforming potential *in vitro* (Hinds et al., 1989). Moreover, it has been shown that mutated p53 could inhibit the activity of wt p53 molecules which made the cells more susceptible to transformation with H-RAS; thus providing an explanation for the confusing data obtained previously by DeLeo et al., Lane et al. and others (Eliyahu et al., 1988). Finally, nearly ten years after *p53* was first identified, its role as a tumour suppressor gene was firmly established (Baker et al., 1989). Intensive research carried out in subsequent years, has led to a

massive increase in our understanding of various aspects of p53 including how it functions and how it is regulated. A brief summary of this will be presented below.

p53 is a 393 AA (amino acid) protein which, has 5 major functional domains (Toledo et al., 2007, Levine, 1997): an amino-terminal transactivation domain (AA 1-42), a proline rich regulatory domain (AA 58-98), a DNA binding domain (DBD AA 102-292), a tetramerization domain (AA 324-355) and a carboxy-terminal regulatory domain (AA 367-393).

p53 functions as a sequence-specific DNA binding protein that can regulate the transcription of target genes (reviewed in Levine, 1997). It has the ability to specifically recognize and bind DNA through a DBD (DNA-binding domain), which coordinates Zn^{2+} ions (Cho et al., 1994). This region recognises and binds to DNA consensus sequences composed of two copies (half-sites) of a palindromic DNA sequence: 5'-PuPuPuC(A/T)(T/A)GPyPyPy 3' (where Pu = purine and Py = pyrimidine) separated with a spacer up to 13bp long (el-Deiry et al., 1992). A more recent, genome-wide search for p53 binding sites, comprehensively describes the p53 binding sequences present in the human genome (Wei et al., 2006). This study quantitatively evaluated representation of each nucleotide in each position of the p53 binding site (see Figure 1.2). In addition, several novel p53 binding sites were identified in promoters of genes which have not previously been reported to be regulated by p53. Interestingly, the study also shows that there are usually no spaces between the half-sites (occasionally, there was one base present in between the half sites). This contradicts the results published by el-Deiry et al. and accords with observations made by (Jordan et al., 2008) who have demonstrated that introduction of the spacer between two half-sites and increasing its length results in dramatically reduced p53-dependent transactivation both in yeast- and human- reporter systems.

Therefore it appears that the presence/length of the spacer between the half-sites determines their functionality (responsiveness to p53). However, the authors showed that half-sites separated by very long spacers or even single half-sites, retain the ability to (weakly) bind p53 and induce transactivation at low levels.

Interestingly, non-canonical p53 binding sites composed of ½ and ¾ of the consensus site (1 or 1½ of the half-site) have also been found to constitute a target for p53 (Jordan et al., 2008); a single ¾ consensus site appeared to induce substantially stronger p53-dependent transactivation than a single half-site.

Since the non-canonical p53-consensus sequences are very abundant in the genome, this finding reveals a possibility, that complexity of the network regulated by p53 may be much higher than previously expected.

The p53 protein acts as a tetramer and all 4 subunits need to be wt for maximal transcriptional activity (reviewed in Joerger and Fersht, 2008). The presence of even one mutant p53 subunit may compromise the activity of the tetramer and this phenomenon is called the dominant negative effect. Mutations of the DBD (exons 5-8) are the most detrimental to the activity of p53 (Hollstein et al., 1991).

p53 is the most frequently mutated tumour suppressor in human cancers and most p53 mutations are situated within its DBD at a frequency exceeding 50% of all cases (reviewed in Vousden and Lu, 2002). Inactivating mutations of the p53 protein results in inability to induce cellular programs such as apoptosis, cell cycle arrest, senescence or DNA repair (see the next section) in response to various types of stresses such as DNA damage or oncogene activation.

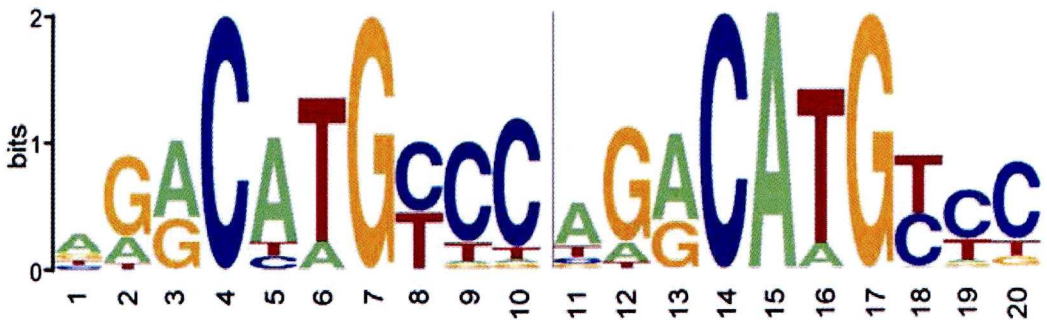


Figure 1.2 **The p53 consensus sequence.** The Figure illustrates two half-sites of the p53 consensus sequence. The letter size in a particular position indicates the frequency with which it is found in p53 binding sites. Reproduced from (Wei et al., 2006).

1.5.2 Functions of p53

p53 functions primarily as a major regulator of apoptosis (reviewed in Vousden and Lu, 2002). When unopposed, p53 acts very potently as it has been shown in mice in which the major negative regulator of p53, Mdm2, has been deleted (Jones et al., 1995); these mice died early in embryonic development due to massive apoptosis. The effect of embryonic lethality appears to be fully rescued by deletion of p53 (Montes de Oca Luna et al., 1995). This observation further supports a critically important role of MDM2 in regulation of p53 and provides additional evidence that p53 plays a major role in induction of apoptosis.

The strong pro-apoptotic activity of p53 makes it a key player in the anti-tumour defence. This has clearly been shown in mice with homozygous deletion of *p53* gene in which it has been demonstrated that as much as 74% of 6 month old $p53^{-/-}$ animals develop tumours and all animals died by the age of 10 months as a result of tumour formation (Donehower et al., 1992, Harvey et al., 1993). For comparison, no wt control animals developed any tumours by the age of 10 months in these studies. Hence, the absence of p53 activity, inevitably results in tumour formation. These results support data obtained from studies of human cancers. Although $p53^{-/-}$ tumours are infrequent, the importance of the intact p53 function is well illustrated by the fact, that mutations of this TSG are present in at least 50% of tumours and the p53 pathway is likely to be compromised in all cancers (Vousden and Lu, 2002).

p53 is a stress-responsive protein; in response to various types of cellular stress such as: DNA damage, oncogene activation, the abnormal presence or absence of survival signals, depletion of ribonucleotides, hypoxia or telomere degradation, p53 becomes stabilised and activated. p53 is then able to mediate a range of cellular processes such

as: apoptosis, senescence, cell cycle arrest or DNA repair (Vousden, 2000). A summary of each of the most important functions of p53 is given below.

1.5.2.1 The role of p53 in apoptosis

Apoptosis is a process of programmed cell death that leads to organized disintegration of the cell in such a way that remaining cellular components may be easily and safely utilised by surrounding cells or phagocytes. Apoptosis is an essential part of development, and is crucial in suppression of tumourigenesis (reviewed in Elmore, 2007). There are several apoptotic pathways in the cell:

- extrinsic (mitochondrial) pathway
- perforin/granzyme pathway
- intrinsic pathway

Despite completely different means of activation, the three apoptotic pathways mentioned above rely on the same mechanism to execute the apoptotic process (Elmore, 2007). Figure 1.3 shows how all apoptotic pathways lead to the activation of caspases (cysteine-aspartic acid proteases) which are responsible for fragmentation of cellular components resulting in cell death.

It has been demonstrated, that p53 has the ability to induce apoptosis by several different mechanisms:

- Activation of the mitochondrial apoptotic pathway by inducing expression of proteins such as BAX, PUMA and NOXA. These proteins increase permeability of the mitochondrial membrane and lead to translocation of cytochrome c to the cytoplasm. This event triggers activation of APAF1 (apoptotic protease activating factor 1) resulting in initiation of the caspase (cysteine aspartate protease) cascade resulting in apoptosis (Cecconi and Gruss, 2001). p53 has also

been shown to induce the mitochondrial apoptotic pathway in a different manner (Chipuk et al., 2005). The process appears to be regulated by two opposing proteins, BCL-xL and PUMA. Sequestered in the cytoplasm by BCL-xL, p53 is inactive. In response to genotoxic stress, nuclear p53 induces PUMA which releases p53 from the complex with BCL-xL. Liberated cytoplasmic p53 may then be translocated to the nucleus or interact with BAX and other members of the BCL-2 family in the cytoplasm to trigger the mitochondrial apoptotic pathway resulting in activation of caspases.

- Enhancing expression of death receptors such as: FAS, PIDD and KILLER/DR5 which could sensitize the cells to death signals and lead to activation of caspases through the extrinsic apoptotic pathway (Vousden and Lu, 2002).
- Activation of FAS and subsequently caspases in a transactivation-independent manner. In a poorly understood way, activated p53 promotes trafficking of FAS to the cell membrane. Once translocated to the outer side of the plasma membrane, FAS can bind FasL (Fas ligand). Ligand binding triggers activation of FAS molecules and transmission of the death signal to the cytoplasm (Bennett et al., 1998).
- Transcriptional activation of the *PTEN* gene by p53 (Stambolic et al., 2001). PTEN functions as a PIP3 phosphatase and, even though dephosphorylation of

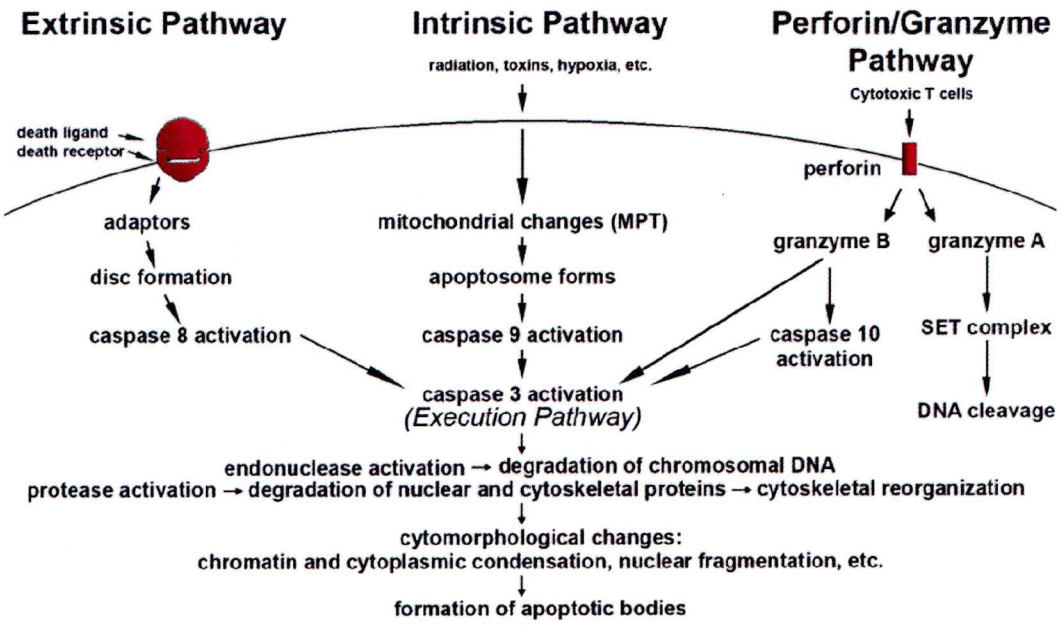


Figure 1.3 Different mechanisms of apoptosis induction. The diagram shows three different apoptosis initiating pathways which lead to activation of apoptosis-related processes, such as DNA degradation, that result in death of the cell. Reproduced from (Elmore, 2007).

PIP3 does not directly lead to apoptosis, it switches off the most important survival pathway which is mediated by AKT, and therefore renders the cell more sensitive to apoptosis induced by the other "conventional" actions of p53 (Mayo and Donner, 2002).

1.5.2.2 The role of p53 in cell cycle arrest

p53 can also induce cell cycle arrest (which is a reversible pause in the cell cycle) in response to cellular stress such as DNA damage. Cell cycle arrest serves to pause the cell during the cell cycle and may allow the cell to repair damage before DNA undergoes replication or before the cell begins to divide. p53 is capable of arresting cells in two main stages during the cell cycle: G1 and G2 (Levine, 1997).

The main transcriptional target of p53 that induces G1 and G2 cell cycle arrest is $p21^{WAF1/CIP1(CDKN1A)}$. p21 mediates its effect by inhibiting cyclinD1-CDK4, cyclinE-CDK2, cyclinA-CDK2 and cyclinA-CDC2 complexes halting cell cycle progression. On the other hand, however, it has been suggested that p21 functions as an adaptor protein promoting association of cyclinD1-CDK4 complex and thus p21 displays a complex interaction with the cell cycle regulatory machinery. It has been shown that, although high levels of p21 inhibit the kinase activity of cyclinD-CDK4 complexes, low levels promote cyclinD-CDK4 complex formation and do not inhibit the kinase activity (LaBaer et al., 1997). Additionally, p21 interacts with PCNA, a DNA polymerase processivity factor, which acts as a cofactor for DNA polymerase delta (Agarwal et al., 1995, Waga et al., 1994). Binding of p21 to PCNA results in a decrease in DNA polymerase processivity thus inhibiting DNA replication and, as a result, also cell division (Levine, 1997). Interestingly this also appears to promote increased DNA repair activity and this is discussed below.

1.5.2.3 The role of p53 in DNA repair

p53 has been shown to induce DNA repair through several different mechanisms, including:

- Binding of the p53-induced p21 to PCNA (mentioned in the previous section) which results in inhibition of DNA replication, however, it does not inhibit DNA repair mediated by this enzyme. This way the cell gains not only a chance to repair damaged DNA, but also an enhanced capacity to do so while the cell cycle is arrested due to action of p21.
- Activation of the *GADD45* (growth arrest and DNA damage 45) gene. It has been shown that GADD45 proteins are essential in maintaining genomic stability, since cells from *Gadd45a*-null mice developed chromosomal rearrangements, amplification of genes and centrosomes and were aneuploid (Hollander et al., 1999). GADD45 takes part in the global genomic repair (GGR), a subtype of nucleotide excision repair (NER) responsible for bases repair, damaged due to action of various environmental factors, such as UV (Friedberg, 2001).

1.5.2.4 The role of p53 in senescence

Senescence is a process in which cells irreversibly enter into a non-proliferating state, their metabolism is decreased and they acquire characteristic morphological changes (Hemann and Narita, 2007). Senescent cells appear large and flat in culture, and therefore this phenotype has been described as resembling a "fried egg" (Wynford-Thomas, 1996). Senescence may be induced in different ways:

- Oncogenic signalling (such as Ras) has been shown to induce senescence through activation of p53 and $p16^{INK4a}$,

- DNA damage and other types of stress have been shown to trigger p53-dependent cellular senescence,
- Telomere erosion results in replicative senescence, which is also dependent on p53 (Wynford-Thomas, 1996, Mooi and Peeper, 2006).

The ability of the cell to induce senescence in response to stimuli mentioned above has several advantages for the host organism, reviewed in (Campisi, 2005, Lowe et al., 2004).

- Protection from cancer, when cells carrying an active oncogene are forced to exit the proliferation cycle,
- Cells with damaged DNA do not replicate which prevents further deterioration of this state (further accumulation of mutations),
- Cells that have reached their replicative limit may still function in the body without posing threat of carcinogenesis.

The description of senescence, presented here, is a simplified one, as it is not essential to discuss it further from the perspective of this project. However, it is worth mentioning that it is still unclear what mechanisms determine which response path is followed for example undergoing senescence instead of, for instance, apoptosis in response to oncogene activation. Senescence appears to be a multi-purpose and multi-factorial process conserved from yeast and worms to humans (Isobe, 2003). As these primitive organisms do not develop cancer, it seems likely that senescence is not only a protection from tumourigenesis, but it remains unclear what the other roles of senescence might be.

1.5.3 Regulation of the p53-MDM2 network

Extreme outcomes of either lack of p53 activity (resulting in tumourigenesis) or unrestrained activity (resulting in death), described in Section 1.5.2 suggests, that p53 must be very tightly regulated in order to provide protection from cancer and allow cell growth and proliferation and sustainable development of the whole organism. Indeed, the process of p53 regulation is complex and precise; any deregulation of this sensitive system may result in unwanted apoptosis or in cancer, (reviewed in Levine, 1997). For instance, a few-fold increase in MDM2 levels caused by SNP T309G was shown to decrease the activity of p53 (Bond et al., 2005) and enhances tumour formation in humans (Bond et al., 2004), also see Section 1.5.3.2.1). On the other hand, it has been demonstrated that animals which express markedly decreased levels of Mdm2 (the mice lack one allele and retain the second, hypomorphic *Mdm2* allele - $Mdm^{puro/-}$) are smaller, radiosensitive and display increased apoptotic rate in several different tissues due to increased activity of p53 (Mendrysa et al., 2003).

p53 is very unstable in unstressed cells and its low level is maintained due to the activity of MDM2. However, in response to various types of stress, p53 becomes stabilized and/or activated. Stabilization and activation of p53 is a very complex, multifactorial process and may be achieved by different means, depending on the type of stimuli and the cellular context (Toledo and Wahl, 2006).

DNA damage signals are key inducers of p53. They are transduced to p53 by members of PI3K family which modify both p53 and MDM2. Phosphorylation of p53 results in stabilization and subsequent activation and may be mediated by ATM (Ataxia - telangiectasia mutated), ATM-activated CHK2 in response to IR (ionizing radiation) and ATR (ATM-related polypeptide) in response to UV mediated DNA

damage. Another kinase, DNA-PK (DNA-dependent protein kinase) is capable of detecting DNA breaks and phosphorylation of p53 (Lakin and Jackson, 1999).

Hypoxia is another type of stress resulting in activation of p53. Prolonged, low levels of oxygen (which often occur in the tumour environment) lead to phosphorylation, stabilisation and accumulation of p53 mediated by ATM and ATR kinases (Hammond et al., 2007). Under hypoxic conditions, p53 is capable of inducing apoptosis (Hammond et al., 2003). To accomplish this, p53 activity has to overcome HIF-1 mediated survival promoting signals which include VEGF (vascular endothelial growth factor) induction and signalling downstream from its receptor (VEGFR) (Schmid et al., 2004). It has been shown that p53 decreases HIF-1α (an unstable subunit of the HIF-1 transcription factor) stability by enhancing MDM2-mediated ubiquitylation of HIF-1α, thereby having an indirect effect on VEGF-mediated vascularisation (Ravi et al., 2000).

Perturbations that occur during DNA replication, such as nucleotide depletion can also lead to induction of p53 (Halazonetis et al., 2008). Interestingly, it has recently been shown that oncogenic activation (activation of p53 in response to activation of oncogenes such as RAS or MYC occurring via E2F-ARF pathway will be described in Section 1.5.3.5) can also result in the same type of cellular response as occurs following nucleotide depletion. Both nucleotide depletion and the activation of certain oncogenes may lead to the division of the cell with incompletely replicated DNA, since either the cell does not have enough substrate to replicate the whole DNA, or the cell cycle progression is too strongly stimulated by an active oncogene. As a result, double-strand breaks (DSB) form which are subsequently detected by CHK2 and ATM, ultimately leading to phosphorylation and activation of p53 (Halazonetis et al., 2008).

Heat-shock is another type of stress that can induce p53 activation, and has been relatively poorly studied. Results from one study, for example, have suggested that it might induce p53 through ATM kinase (Miyakoda et al., 2002), but another study has demonstrated that ATM is not essential for p53 stabilization in response to heat-shock and that the mechanism of p53 activation involves interaction of p53 with the HSP90 chaperone (Wang and Chen, 2003). Other members of the HSP family, HSP70 and HSP27, have also been shown to regulate p53 in response to heat-shock (Hinds et al., 1987, Gao et al., 2000). Interestingly, the expression of both HSP70 and HSP27 seems to be regulated by p53 suggesting the existence of a feedback loop between p53 and HSP proteins (Gao et al., 2000, Agoff et al., 1993).

Oxidative stress has also been shown to modulate functions of p53. The p53 protein undergoes post-translational modifications such as phosphorylation by p38 (Bragado et al., 2007), ERK (Persons et al., 2000) or ATM (Kurz and Lees-Miller, 2004) in response to elevated ROS, resulting in activation of p53. Interestingly, the thiol (-SH) groups of p53 which are present in the DNA binding site, have been shown to constitute direct targets of ROS (Hainaut and Mann, 2001) and appeared to modulate the DNA binding ability of p53 and this also contributes to the direct regulation of p53 by oxidative stress (reviewed in Liu et al., 2008).

1.5.3.1 Post-translational modifications of p53

The p53 protein undergoes various post-translational modifications that may affect its stability and function (reviewed in Prives, 1998). Figure 1.4 illustrates most of the known post-translational modifications of p53 occurring in response to stress. Several residues of p53 have been shown to be modified upon stress by phosphorylation, acetylation, ubiquitylation, SUMOylation or NEDDylation.

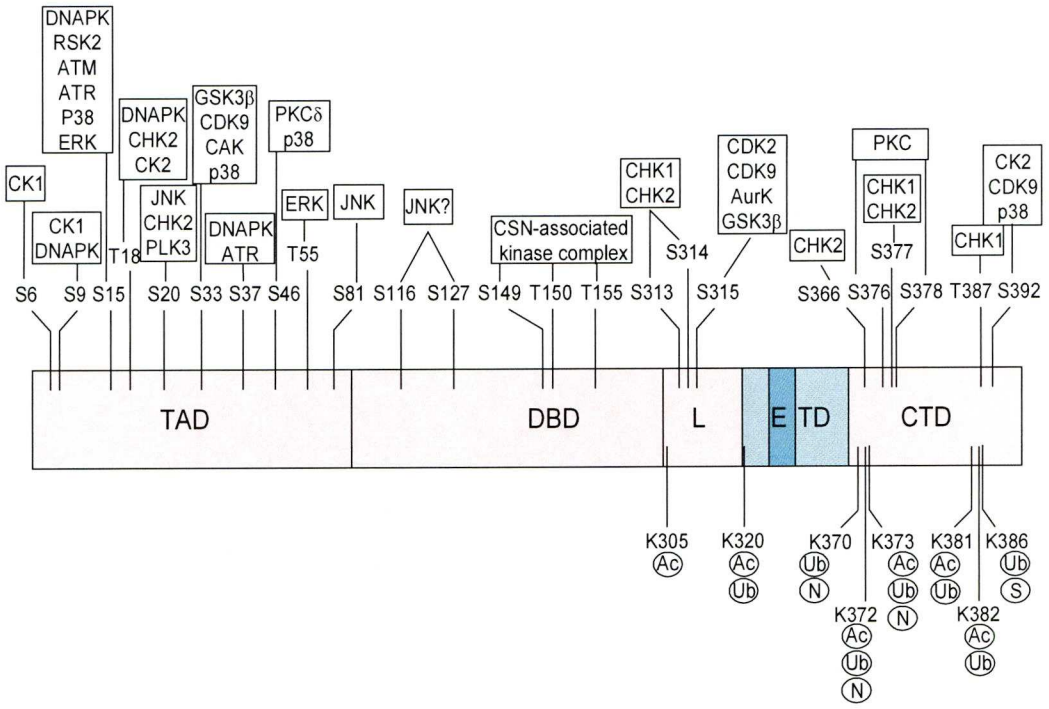


Figure 1.4 **Modifications of p53.** The Diagram shows most of the known modifications of p53 within its transactivation domain (TAD), DNA binding domain (DBD), nuclear localization sequence (L), tetramerization domain (TD) and the carboxy-terminal regulatory domain (CTD). The letter "E" denotes the nuclear export signal sequence. Each phosphorylation site constitutes a target for at least one kinase, which is indicated above. Modifications of lysines of the carboxy-terminal domain are shows at the bottom of Figure and are presented as: Ac (acetylation), Ub (ubiquitylation), N (NEDDylation) and S (SUMOylation) (Toledo and Wahl, 2006, Bode and Dong, 2004).

Several lines of evidence (mostly *in vitro* studies) suggest that post-translational modifications (excluding ubiquitylation which plays a critical role in p53 turnover and will be described in the following section) play important roles in regulation of the activity and stability. For instance, phosphorylation of S15 has been shown to result in stabilisation of p53 resulting in apoptosis, reviewed in (Bode and Dong, 2004). Interestingly, however, S18A mutant of p53 expressed in mice (S18 of murine p53 is an equivalent of S15 in human p53) appeared to have normal stability and ability to induce cell cycle arrest and only partially defective apoptotic response to DNA damage (Sluss et al., 2004). This appears to be true also for other types modifications studied *in vivo* these modifications, reviewed in (Toledo and Wahl, 2006). Therefore, unlike the major regulators of p53 such as MDM2, MDMX and ARF (described below), post-translational modifications have been suggested to play a role in fine-tuning the activity of p53.

1.5.3.2 MDM2 as a major negative regulator of p53

Mdm2 (murine double minute 2) was identified as one of three genes found in extra-chromosomal nuclear bodies called "double minutes" of a spontaneously transformed murine cell line – 3T3-DM (Cahilly-Snyder et al., 1987). Since only Mdm2 manifested oncogenic activity by inducing tumourigenicity of transfected cells (Fakharzadeh et al., 1991), it became a target of extensive investigation. It has subsequently been discovered that MDM2 functions as a major negative regulator of p53. The following sub-sections will summarise the current knowledge regarding how MDM2 executes its p53-suppressive functions.

1.5.3.2.1 Clinical evidence for the MDM2-dependent inhibition of p53

MDM2 was first identified as a p53-binding protein in co-precipitation experiments (Momand et al., 1992). This study also showed, for the first time, that MDM2 was capable of reducing the transcriptional activity of p53. Subsequently, several different studies have shown that amplification of the *MDM2* gene (leading to over-expression of the MDM2 protein) was present in a subset of soft tissue sarcomas sustaining wt p53.

Failure to detect p53 mutations in an MDM2-over-expressing group of tumours suggests that over-expression of MDM2 itself may be sufficient to compromise the p53 pathway (Leach et al., 1993, Oliner et al., 1993, Cordon-Cardo et al., 1994). Several lines of evidence suggest that over-expression of MDM2 may result in tumourigenesis due to inhibition of p53-mediated tumour suppression. Increased expression of MDM2, similarly to soft tissue sarcomas, has been observed in various other types of cancers, carrying wt p53, such as: gastric, kidney, non- Hodgkin's and Hodgkin's lymphoma and leukeamias (Ohmiya et al., 2006, Haitel et al., 2000, Maestro et al., 1995, Chilosi et al., 1994, Lu et al., 2002, Bueso-Ramos et al., 1993). Additionally, the contribution of increased levels of MDM2 to tumour development has been demonstrated in Li-Fraumeni patients carrying a SNP309 (309T>G polymorphism) in the MDM2 gene where thymidine in the position 309 of *MDM2* gene is substituted by guanosine. The T>G nucleotide substitution has been shown to result in increased expression of MDM2 as it creates an enhanced SP1 transcription factor binding site in the promoter of the gene (Bond et al., 2004). Li-Fraumeni patients who carried SNP309 displayed much earlier onset of tumours, with a mean of 29.7 years of age compared with 45.5 years in the group of patients who had T in position 309 (Ruijs et al., 2007). Since it is unlikely that the frequency of

mutation/loss of the second allele was higher in the SNP309 group of patients (even if the cells growing in the tumour eventually lose the second p53 allele), it is suggested that elevated levels of MDM2 inhibit the ability of p53 to suppress tumourigenesis more efficiently, which results in significantly earlier onset of tumours.

1.5.3.2.2 How does MDM2 antagonize p53?

There are three major ways in which MDM2 can inhibit p53: by promoting p53 degradation through ubiquitin-mediated proteolysis, promoting p53 nuclear export by mono-ubiquitylation and by inhibiting p53 transcriptional activity through direct binding, reviewed in (Freedman et al., 1999). These antagonistic activities of MDM2 towards p53 are described in more detail below.

MDM2 functions as a homodimer or a heterodimer with its homolog, MDMX (Stad et al., 2001) and section 1.5.3.4. MDM2 has been found to possess E3 ubiquitin ligase activity within its RING-finger domain that catalyses covalent attachment of ubiquitin (Ub) molecules with the result that the ubiquitylated p53 substrate can be targeted for proteasomal degradation. This p53 degradation may occur in the nucleus or in the cytoplasm (Joseph et al., 2003). p53 has been shown to be one of the major targets for the E3 ligase activity of MDM2 and therefore MDM2 functions as a negative regulator of stability of the p53 protein (Honda et al., 1997) and Section 1.4.4. The ability of Mdm2 to promote degradation of p53 appears to be essential for mouse development, as mice that express only the Mdm2-RING-finger mutant, lacking the E3 ubiquitin ligase activity (*Mdm2-C462A* knockin mice), die before day E7.5 of embryonic development and this phenotype can be rescued by deletion of p53 (Itahana et al., 2007).

MDM2 can mediate both mono- and poly-ubiquitylation of p53. Whilst poly-ubiquitylation usually results in degradation of the target protein, mono-ubiquitylation does not lead to degradation, but can modulate the activity of proteins (Maguire et al., 2008) or their ability to interact with other proteins (Brooks et al., 2004). Mono-ubiquitylation of p53 by MDM2 has been shown to promote nuclear export of p53 which prevents p53 from playing its role as a transcription factor (Li et al., 2003). More interestingly, it has recently been demonstrated that mono-ubiquitylation of p53 uncovers the NES and also facilitates the carboxy-terminal SUMOylation of p53 resulting in decreased MDM2-p53 interaction and enhanced nuclear export of p53 (Carter et al., 2007). The mechanisms that mediate the process of p53 translocation to the cytoplasm remain unknown. It is also unclear what might be the function of the cytoplasmic p53. It has been speculated that cytoplasmic p53 might be involved in some transactivation-independent activities such as FAS activation (Sigal and Rotter, 2000), translocation to the mitochondria and alternative p53 pathways leading to the induction of apoptosis (Zhao et al., 2005, Mihara et al., 2003) or even more enigmatically the process of binding to ribosomes (Fontoura et al., 1997). Another possibility is that mono-ubiquitylation may serve as a means of creating the energy-saving reservoir of a transcriptionally inactive p53 (it is inactive because it is localized in the cytoplasm), that can be quickly reactivated by transporting it back into the nucleus if stress signals appear (Brooks et al., 2004).

Another inhibitory effect of MDM2 on p53 is mediated by direct interaction between the two proteins. MDM2 has been shown to bind strongly to the transactivation domain of p53 and to form a complex with p53 in cells resulting in potent inhibition of transcriptional activity of p53 (Oliner et al., 1993, Momand et al., 1992). It has been demonstrated that this interaction is independent of the RING finger of MDM2

as the RING finger mutant of MDM2 appeared to be as potent an inhibitor of transcriptional activity of p53 as wt MDM2 protein in p53 reporter assays and has also been shown to have stimulatory effect on cell cycle progression (Argentini et al., 2000). However, as mentioned previously, mice expressing only the Mdm2-RING-finger mutant (instead of the wt protein) die during early embryonic development and this lethal phenotype can be rescued by concomitant deletion of p53. This result suggests that binding and repression of p53 transcriptional activity in the absence of E3 ubiquitin ligase activity of Mdm2 is not sufficient to inhibit the proapoptotic activity of p53 in developing embryos (Itahana et al., 2007).

A number of cellular factors have been shown to modulate the efficiency of MDM2-mediated ubiquitylation of p53 and binding of MDM2 to p53. Post-translational modifications of p53 and MDM2, as well as interactions with other proteins (described later in this section), allow multi-step control of the p53-MDM2 network, which is critical for maintenance of cellular homeostasis.

Similarly to p53, also MDM2 undergoes post-translational modifications which modulate its ability to ubiquitylate target proteins. Apart from ubiquitylating p53, MDM2 also has the ability to ubiquitylate itself *in vitro*, thus regulating its own turnover (Fang et al., 2000). The E3 ligase activity of MDM2 is encoded by the RING finger domain and it has been demonstrated that the RING finger mutants of MDM2 were more stabile, most likely due to the absence of auto-ubiquitylation (Honda et al., 1997, Fang et al., 2000). Interestingly, although the ability of MDM2 to auto-ubiquitylate *ex vivo* is well documented and appears to play a major role in regulation of MDM2 protein levels, *in vivo* studies have revealed that the E3 ubiquitin ligase activity of Mdm2 is not essential for Mdm2 degradation. A RING-finger mutant of Mdm2 expressed in early passage MEFs derived from *Mdm2-*

C462A knock-in animals, appeared to display a steady state level and turnover indistinguishable (half-life around 20 min.) from that of the endogenously expressed wt protein. Moreover, the half-life of Mdm2 appeared to decrease equally in MEFs expressing wt and RING-finger mutant Mdm2 after exposure to IR (Itahana et al., 2007 437). It has therefore been proposed that auto-ubiquitylation is not required for Mdm2 degradation under physiological conditions, suggesting the existence of some, yet unidentified factors regulating ubiquitylation and level of the Mdm2 protein.

As stated earlier MDM2 has also been shown to be phosphorylated by several kinases such as DNA-PK (Mayo et al., 1997), ATM (Maya et al., 2001) or ATR (Shinozaki et al., 2003) which appeared to impair the ability of MDM2 to promote p53 turnover. However, MDM2 has also been shown to be phosphorylated by AKT, which was shown to promote stabilization and nuclear localisation of MDM2 and, subsequently, enhanced inhibition of p53 (Mayo and Donner, 2001, Ogawara et al., 2002, Zhou et al., 2001).

It has also been demonstrated that MDM2 undergoes acetylation which has been shown to be mediated by CBP and to a lesser extent also p300 histone acetyl transferases (Wang et al., 2004). Acetylation of MDM2 appeared to occur within its RING-finger domain and was shown to inhibit the MDM2-dependent degradation of p53. On the other hand, other types of post-translational modifications such as SUMOylation (Buschmann et al., 2000) or NEDDylation (Watson et al.) have been shown to stabilise MDM2 and promote the MDM2-dependent inhibition of MDM2. However, these types of modifications of MDM2 and their functional consequences are still not comprehensively described; therefore they will not be discussed further.

1.5.3.3 The p53-MDM2 auto-regulatory feedback loop

There are several ways in which the levels and activity of both p53 and MDM2 can be regulated, as described in previous sections. However, the discovery of the p53-responsive element in the *MDM2* promoter shed new light on their regulation. It has been demonstrated that p53 can regulate its own degradation by enhancing the expression of MDM2, thus creating a negative feedback loop between these two molecules (Wu et al., 1993).

In the absence of stress stimuli or in the presence of growth/proliferation signals, p53 levels are maintained low due to MDM2-dependent degradation. The stress responses disturb the auto-regulatory feedback loop by induction of the post-translational modifications of p53 and MDM2. Most of these prevent the protein-protein interaction and protect p53 from the MDM2-mediated degradation (Freedman et al., 1999).

The ability of MDM2 to negatively regulate p53 makes it a potent oncogene. As described earlier, MDM2 promotes degradation, cytoplasmic shuttling and inhibition of p53-dependent transactivation, reviewed in (Vogelstein et al., 2000). Several different mechanisms govern the MDM2-p53 interplay, with profound effects on the functions of both proteins. Several types of post-translational modifications (mentioned in the present section) in response to different internal and external stimuli (existence or absence of growth signals, DNA damage, hypoxia, oncogene activation etc.) modulate the activity of this sensitive and multifactorial system and allow the cell to grow and proliferate, but also maintain control over the cell cycle and genetic stability. The following sections will focus on other components of the p53-MDM2 network playing important roles in its regulation.

1.5.3.4 The role of MDMX in regulation of p53 and MDM2

Mdmx (also known as Mdm4) protein was initially discovered in a screen of a mouse expression library with radioactively labelled p53 used as a bait protein (Shvarts et al., 1996). Subsequently, the human homologue (HDMX), exhibiting 90% amino acid identity with its murine counterpart, has been identified and described (Shvarts et al., 1997). The amino acid sequence of MDMX was also found to be similar to the sequence of MDM2, hence the name. The highest degree of homology, present within the p53 binding domain, suggests an important functional role for this region (Shvarts et al., 1997). A high degree of homology has also been found between the RING domains of MDM2 and MDMX through which the proteins can heterodimerize, reviewed in (Marine et al., 2006), however, it has been shown that MDMX does not possess E3 ubiquitin ligase activity, reviewed in (Marine and Jochemsen, 2005). Extensive studies have revealed that, although MDMX does not directly promote p53 degradation, it reduces the activity of p53 through binding to its transactivation domain (Shvarts et al., 1996, Jackson and Berberich, 2000, Little and Jochemsen, 2001). In addition, MDMX has also been proposed to regulate p53 activity through inhibition of p53 acetylation by p300/CBP (both in presence and absence of MDM2) (Danovi et al., 2004, Sabbatini and McCormick, 2002). The ability to regulate acetylation of p53 has been shown to be dependent on the p53-binding domain of MDMX which suggests that binding of MDMX to p53 is essential for inhibition of the p300/CBP-mediated acetylation of p53 (Danovi et al., 2004). This provides therefore an obvious explanation for the apparently greater ability of MDMX to act as an inhibitor of p53 transcriptional activity.

In contrast with the well established role of MDMX as a suppressor of p53 activity, it remains unclear, what is the involvement of MDMX in regulation of the levels of

p53 and MDM2. Several studies have revealed a potential significance of MDMX expression levels and the relative levels between the level of MDMX and MDM2 in regulation of p53. High levels of MDMX appear to compete with MDM2 and MDM2-MDMX dimers for binding and degradation of p53; as a result, p53 becomes upregulated (Sharp et al., 1999, Stad et al., 2000, Stad et al., 2001). On the other hand, depletion of MDMX has been observed to result in increased levels of p53 (Linares et al., 2003) as heterodimerization of MDM2 and MDMX has been shown to be vital for stabilization of MDM2 which enhances degradation of p53 (Gu et al., 2002). It is therefore likely that the ratio between the MDMX and MDM2 expression levels is critical for precise regulation of the levels of both p53 and MDM2.

Analysis of MEFs from either *Mdm2* or *Mdmx* knockout mice, conditionally expressing of p53 (a non-conditional knockout of *Mdm2* or *Mdmx* is lethal) revealed that, unlike the loss of *Mdm2*, loss of *Mdmx* does not seem to significantly alter the levels of p53, as deletion of both *Mdm2* and *Mdmx* resulted in expression of similar p53 levels as in the $Mdm2^{-/-}$ MEFs (Francoz et al., 2006). Analysis of the transcriptional activity of p53 in this system has revealed that restoration of p53 in the $Mdmx^{-/-}$ MEFs increased activity of p53 only slightly (compared to the $Mdmx^{+/+}$ background) which contrasted with deletion of *Mdm2* which resulted in a very dramatic increase of p53 activity (compared to the $Mdm2^{+/+}$ background). This phenomenon has been explained by the activation of p53-MDM2 feedback loop in the absence of negative regulation of p53 by Mdmx. As a result, increased levels of Mdm2 would partially compensate for the absence of Mdmx leading to a decrease of the activity of p53. Further clues supporting the idea that Mdm2 and Mdmx play different roles in the regulation of p53, came from *in vitro* studies performed on *Mdm2/Mdmx*-null MEFs expressing a temperature sensitive mutant of p53 (the wt

activity of the temperature sensitive mutant of p53-A135V is switched on by increasing the temperature to 39°C in the absence of either Mdm2 or Mdmx). It has been demonstrated that deletion of *Mdmx* results in increased, p53-mediated expression of cell cycle arrest genes such as p21, as well as Mdm2. In contrast, deletion of *Mdm2* has been shown to increase expression of apoptosis-mediating genes, such as *Bax* and *Noxa* (Barboza et al., 2008) strongly suggesting that Mdm2 and Mdmx regulate the transcriptional repertoire of p53 in different ways and may provide distinct modes of inactivation of p53 function.

It has been proven that Mdmx is essential for embryonic development; depending on the type of mouse model, deletion of both *Mdmx* alleles led to early embryonic lethality around day E10.5 (Migliorini et al., 2002, Finch et al., 2002). This phenotype could be rescued by concomitant knockout of p53 (Parant et al., 2001) thus proving the importance of Mdmx in regulation of p53 activity.

In contrast, investigation of the tissue-specific p53-suppressive functions of Mdmx revealed a striking difference in regulation of p53 by Mdm2 and Mdmx. Homozygous deletion of *Mdmx* in individual tissues did not result in embryonic lethality. It has been demonstrated that conditional deletion of *Mdmx*, in smooth muscle cells of the gastrointestinal tract, had no apparent effect in this tissue (Boesten et al., 2006). Similar results have been obtained when the *Mdmx* gene has been conditionally knocked out in cardiomyocytes. However, the animals that had the MDMX gene knocked out in their cardiomyocytes, died prematurely later on due to unknown condition/s (Grier et al., 2006). Both studies show a strong discrepancy between the actions of Mdmx and Mdm2, as deletion of the *Mdm2* in the same tissues resulted in lethality of the animal. Similarly to the conclusions drawn by Francoz et al. based on *in vitro* studies, it has been suggested that loss of Mdmx can

be compensated by increased levels of Mdm2 in these tissues, especially as the knockout of *Mdmx* has been shown to increase p53-driven expression of Mdm2. On the other hand, knockout of *Mdm2* has been shown to result in a massive accumulation of p53 whose activity could not be efficiently controlled by the remaining Mdmx (reviewed in Marine et al., 2006).

Similarly to MDM2, over-expression or amplification of MDMX has been shown to mediate oncogenic effects and it this has been observed in several types of malignancies such as gliomas (Riemenschneider et al., 2003) or breast cancers (Ried et al., 1995). Moreover, reduction of the MDMX protein levels using RNAi in the MCF-7 cell line (which over-expresses MDMX), has been demonstrated to reduce the number of clones obtained in a colony formation assay (Danovi et al., 2004). The same study has shown that over-expression of MDMX can cooperate with oncogenic RasV12 in transformation of MEFs which, as a result, acquire oncogenic properties .

To sum up, MDMX presents itself as an essential for life, negative regulator of p53. Both laboratory and clinical data suggest that increased levels of MDMX mediate tumourigenesis. At the molecular level, MDMX has been shown to bind to, and inhibit the activity of p53 both *in vitro* and *in vivo*. It is still unclear what is the role of MDMX in regulating the levels of p53 and MDM2. Although studies on animal models suggest that Mdmx rather inhibits the p53-Mdm2 feedback loop by decreasing the activity of p53, more studies addressing these issues are needed to unravel features of the p53-MDM2-MDMX network.

1.5.3.5 The role of ARF in regulation of p53 and MDM2

The $p14^{ARF}$ tumour suppressor is encoded in an alternate reading frame (hence the name) of the *INK4a* locus which also encodes the tumour suppressor protein,

$p16^{INK4a}$ (reviewed in Sherr, 2000). Expression of ARF has been shown to be differentially regulated in response to oncogenic/mitogenic signalling and appears to be dependent on the E2F transcription factor. Oncogenic activation of proteins such as RAS and MYC, which stimulate the activity of E2F, or presence of some viral oncogenes has been reported to increase expression of ARF (Sherr, 2000).

ARF has been shown to bind MDM2 which led to inhibition of MDM2-mediated ubiquitylation of p53 resulting in stabilization of the latter one, reviewed in (Sherr and Weber, 2000, Vousden, 2000). However, as will be discussed below, Arf has also been shown to alter cellular localization of MDM2 in some types of cells and several different mechanisms of this phenomenon have been proposed, see Figure 1.5. In cells unaffected by oncogenic stress the level of Arf is usually low, both p53 and MDM2 localize in the nucleoplasm and p53 turnover is very rapid. In model one, depicted in Figure 1.5 (a) it has been proposed that binding of Arf to MDM2 leads to nucleolar sequestration of MDM2 and activation of p53, at least in some cells. It has been demonstrated that up-regulated Arf (but not Arf mutant which fails to localize to the nucleolus) relocalizes MDM2, but not p53, to the nucleolus. It has also been shown that mutant of Arf unable to bind MDM2, fails to relocalize MDM2 to the nucleolus and to trigger p53 response suggesting a potential role of MDM2 shuttling between nucleus and nucleolus in regulation of p53 (Weber et al., 1999). This model also suggests that blocking the nuclear export of p53 might also contribute to Arf-mediated activation of p53.

Another model depicted in Figure 1.5 (b) predicts that cytoplasmic shuttling of MDM2 occurs, at least in part, via the nucleolus. Using heterokaryon assays, it has been demonstrated that MDM2 gradually accumulates in the nucleolus where it co-localizes with Arf (Tao and Levine, 1999). The model suggests that the nuclear

export of MDM2 through the nucleolus could constitute a barrier for the nuclear export of p53 when Arf becomes induced. Interestingly, the models (a) and (b) were created based on the results obtained in different types of cells. Weber et al. used MEFs whereas Tao et al. used MEFs-derivative cells fused with human cervical cancer HeLa cells. This creates a potential source of variations, such as presence of oncogenic stress or DNA damage signals in HeLa cells, and could potentially contribute to the differences in observations in the two studies and differences between these two models.

In contrast with the two previous models, which examined murine ARF protein, the model shown in Figure 1.5 (c) has been proposed based on research carried out on human ARF. It has been shown that p53, MDM2 and ARF accumulate in the nucleoplasm in nuclear bodies resulting in inhibition of the nuclear export of both p53 and MDM2, accompanied by stabilization of p53. Interestingly, human ARF has been shown to localize predominantly in the nucleoli of cells expressing low levels of MDM2. However, over-expression of MDM2 results in nucleoplasmic localization of ARF in several different cell lines. Moreover, it has been demonstrated that relocalization of ARF from the nucleolus to the nucleoplasm (where it co-localizes with MDM2 in the nuclear bodies) was induced by oncogenic signalling. (Zhang and Xiong, 1999). This has been observed in both cancer and fibroblast-derived human cell lines and contrasts with the results obtained in murine cells using murine Arf. This suggests a potentially different mode of ARF-mediated inhibition of MDM2 in mice and humans.

Apart from the mechanisms of ARF-dependent regulation of p53 and MDM2, presented above, described in (Sherr and Weber, 2000), other possible ways of MDM2 and p53 regulation by ARF have also been proposed. ARF has been

suggested to prevent inhibition of the transactivation domain of p53 by MDM2. In support of this idea, it has been shown that binding of ARF to MDM2 resulted in increased transcriptional activity of p53 (Pomerantz et al., 1998). It has also been shown that ARF decreases the E3 ubiquitin ligase activity of MDM2 towards p53 (Honda and Yasuda, 1999) and as a consequence of this, ARF appears to inhibit the MDM2-dependent degradation of p53. This way of action of ARF seems to be a generally accepted paradigm (reviewed in Sherr, 2006) and is not mutually exclusive with other possible or putative mechanisms of ARF-dependent regulation of p53 presented above.

The ability of ARF to rescue p53 from MDM2-mediated inhibition in response to oncogenic signalling has been shown to be an important anti-tumourigenic mechanism. The role of ARF as a tumour suppressor gene has been supported by the fact that inactivating mutations in the *ARF* gene have been found in various types of tumours (Pollock et al., 1996). In addition, 80% of *Arf* null mice developed tumours (predominantly lymphomas and sarcomas) with a mean latency of 38 weeks (Kamijo et al., 1999). Although the tumours retained wt p53, suggesting that deletion of *Arf* is sufficient to compromise the response of p53 to oncogenic stress (hence, tumours developed), different tumour spectrum as well as delayed tumour latency compared with *p53*-null mice (mean latency 18-20 weeks) has been observed. It has been suggested that the ability of p53 to respond to other types of stress (such as DNA damage) could contribute to a residual p53 activity in *Arf*-null cells resulting in development of tumours later than in *p53*-null animals (Lozano and Zambetti, 2005).

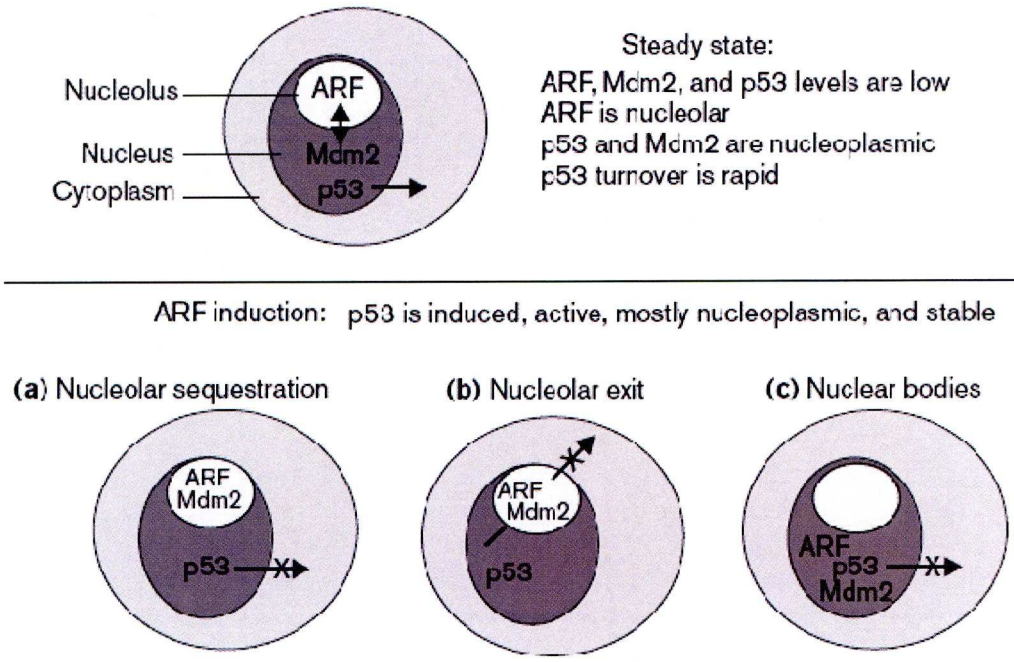


Figure 1.5 **ARF-mediated activation of p53.** Reproduced from (Sherr and Weber, 2000).

The importance of Arf in activation of the p53 pathway has been further demonstrated by analysis of lymphomagenesis model mice (in which over-expression of the c-Myc oncogene is driven by the immunoglobulin heavy chain enhancer). Thess studies have revealed that 28% of lymphomas that arise had sustained loss of function of p53, 24% loss of function of Arf and the rest of the tumours over-expressed Mdm2. This suggests that all three situations lead to a similar outcome - inactivation of the p53 pathway. Moreover, c-Myc over-expressing $Arf^{+/-}$ animals appeared to have markedly reduced survival compared to the $Arf^{+/+}$ mice and 80% of lymphomas developed in these mice appeared to have lost the second *Arf* allele. The c-Myc over-expressing, $Arf^{-/-}$ animals died of lymphomas a few weeks after birth, further supporting the importance of Arf in providing defence against oncogenic activation (Eischen et al., 1999).

1.6 MDM2 has p53 independent functions and interactions

The data described in previous sections strongly suggest that MDM2 functions as an oncogene, as it leads to suppression of the activity of p53. However, MDM2 appears to have also p53-independent interactions which may contribute to oncogenesis. Some of these are shown in the Figure 1.6.

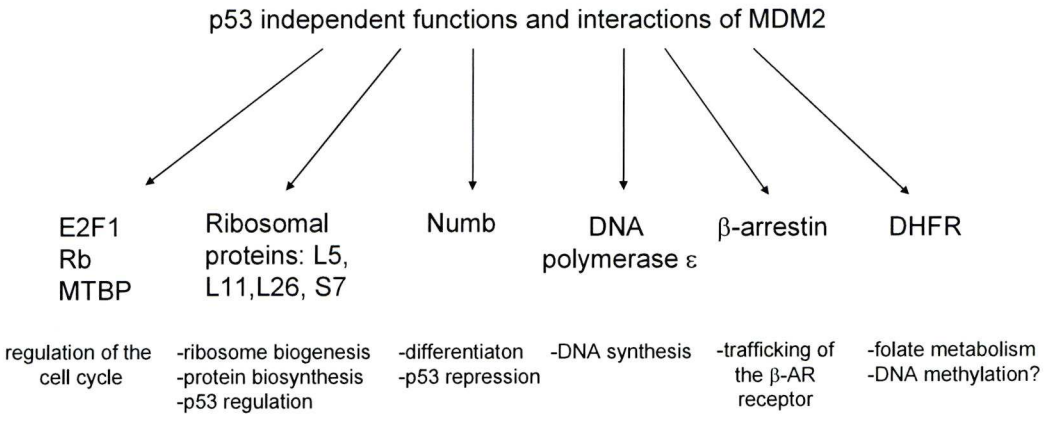


Figure 1.6 **p53-independent targets of MDM2**. The Diagram presents various proteins that are bound by MDM2 and target proteins/mechanisms which are regulated by these interactions, adapted from (Iwakuma and Lozano, 2003).

MDM2 is known to be involved in various cellular processes.

- MDM2 has been shown to interact with cell cycle regulators. Inhibition and ubiquitylation of the tumour suppressor Rb by MDM2 has been shown to result in E2F-dependent cell cycle progression and loss of Rb dependent G1 arrest (Uchida et al., 2005). Interaction of MDM2 with E2F1 heterodimeryzation partner, DP1, has been demonstrated to increase activity of the E2F1-DP1 heterodimer promoting transactivation of the S-phase genes (Martin et al., 1995). MDM2 has also been shown to inhibit the G1 cycle arrest mediated by MTBP (Boyd et al., 2000).
- MDM2 has been implicated in regulation of protein synthesis and ribosome biogenesis through binding of several ribosomal proteins. Moreover, it has been reported that interaction with several ribosomal proteins, such as L11 (Zhang et al., 2003) or S7 (Chen et al., 2007) has leads to stabilization of p53 and increase p53 activity.

- MDM2 has been shown to have an effect on differentiation through interaction with Numb, a negative regulator of Notch signalling. This interaction results in ubiquitylation and proteasomal degradation of Numb (Yogosawa et al., 2003).
- MDM2 is also known to be involved in regulation of DNA synthesis due to interaction with carboxy-terminus of the DNA polymerase ε which results in increased activity of the enzyme (Vlatkovic et al., 2000, Asahara et al., 2003).
- MDM2 has been found to regulate trafficking and turnover of the β2AR. By ubiquitylation and degradation of β-arrestin, a key negative regulator of the β2-adrenergic receptor (β2AR) responsible for regulation of its internalization, MDM2 leads to internalization and sequestration of the β2AR (Ganguli and Wasylyk, 2003).
- MDM2 has also been implicated in folate metabolism through interaction with and mono-ubiquitylation of dihydrofolate reductase DHFR (an enzyme catalysing regeneration of tetrahydrofolate, essential for thymidine synthesis, and therefore also for DNA replication). Inhibition of DHFR by MDM2 has been shown to result in decreased activity (but not stability) of DHFR. It has been suggested that apart from compromising the ability to regenerate tetrahydrofolate, inhibition of DHFR by MDM2 may also play a role in epigenetic regulation by methylation, thus potentially contributing to tumourigenesis (Maguire et al., 2008).

In spite of extensive studies addressing the issue of p53-independent functions of MDM2 and their potential contributions to cancer, very little is known about the molecular basis of these MDM2-mediated processes. However, there is a considerable body of evidence, such as clinical and animal model data, suggesting that in addition to inhibiting p53, MDM2 also possesses p53-independent oncogenic

activities. For example, it has been shown that increased levels of MDM2 expression in addition to mutation of p53, correlate with decreased survival of bladder cancer patients (Lu et al., 2002). Analysis of the $p53^{-/-}$ mice over-expressing Mdm2 has demonstrated a different spectrum and incidence of tumours in these mice compared with $p53^{-/-}$ animals. For example, $p53^{-/-}$ mice over-expressing Mdm2 suffered from increased incidence of sarcomas (38%) compared with *p53* null animals (9%). This suggests a p53-independent role for Mdm2 in pathogenesis of this particular group of tumours (Jones et al., 1998). Another study, in which Mdm2 has been over-expressed in the mammary gland during gestation and lactation showed poor development of the gland and perturbations of the cell cycle in mammary epithelial cells in both *p53* wt and $p53^{-/-}$ mice. The cells from both $p53^{-/-}$ and wt animals appeared to undergo multiple S-phases without mitotic divisions. Moreover, 16% of *p53* wt, Mdm2-over-expressing mice developed mammary adenocarcinomas due to over-expression of Mdm2 after a long (14-18 months) period of latency. However, it remains unclear whether this is due to p53-independent activity of Mdm2, or by Mdm2-mediated inhibition of p53 (Lundgren et al., 1997). Additional evidence for the p53-independent role of Mdm2 in tumourigenesis has come from studies of Mdm2 deletion mutants (Fridman et al., 2003). Mutants of Mdm2 used in this study represented the splice variants of Mdm2 expressed in human lymphomas. This study has revealed that mutants of Mdm2, which are unable to bind p53, also contribute to development of lymphomas (similarly to the wt Mdm2 protein) in mice. In addition, a different study has demonstrated that splice variants of Mdm2 that do not have the ability to bind p53 still retain the transforming potential (in combination with oncogenic Ras) (Sigalas et al., 1996).

1.7 Aim of the project

Several lines of evidence suggest the existence of p53-independent oncogenic activities of MDM2 (see Section 1.6). In renal cell carcinoma, concomitant over-expression of both p53 and MDM2 appears to be associated with a worse outcome than over-expression of p53 alone (see Section 1.4). This suggests a possible functional role for MDM2 in promoting aggressiveness of RCC tumours and makes RCC an excellent model to study p53-independent oncogenic functions of MDM2. Therefore, we decided to search for novel oncogenic functions of MDM2 in RCC cells.

Since MDM2 executes essentially all of its known functions *via* protein-protein interactions, this project aimed to identify novel protein targets of MDM2. Identification of novel MDM2-binding proteins specific for RCC was hypothesised to be likely to explain the role of MDM2 in mediating the aggressive phenotype of this type of cancer.

An additional aim was to characterize RCC cells over-expressing both p53 and MDM2 with respect to their p53 status and functionality as this might contribute to our understanding of the regulation of p53 and MDM2 levels in RCC. Finally, since increased cell motility and invasiveness contribute to the metastatic process (motile cells can actively disseminate from the primary tumour and migrate to other tissues), the role of MDM2 in promoting these cellular activities will also be investigated in the present study.

2 Materials and methods

2.1 List of reagents

Reagent	Manufacturer
3-amino-1,2,3-triazole	Fluka
Acrylogel 2,6 (40%) solution	Electran
Adenine hemisulfate salt	Sigma
Agar	Oxoid
Agarose	Flowgen bioscience
Agarose Seakem GTG	Cambrex
Albumin, bovine	Sigma
Amonnium peroxodisulfate	BDH
Ampicillin sodium salt	Sigma
Aprotinin	Roche
Bio-rad protein assay reagent	Bio-rad
Blotting grade blocker, non-fat dry milk	Bio-rad
Bromophenol blue	Sigma
BSA (bovine serum albumin)	Sigma
Chloroform	BDH
D(+)-glucose solution	Sigma
Dimethylformamide	BDH
DMSO (dimethyl sulfoxide)	Sigma
Dulbecco's modified eagle's medium	Sigma

Dulbrcco's phosphate buffered saline	Sigma
EDTA (ethylenediamine tetraacetic acid)	Sigma
Ethanol absolute for molecular biology	Sigma
Ethidium bromide solution	Sigma
Fetal Bovine Serum	Sigma
Formaldehyde solution 37%	BDH
Gene juice	Novagen
Glass beads	Sigma
D-(+)-glucose	Formedium
Glycerol form molecular biology	Sigma
Glycine	BDH
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Calbiochem
Hybond ECL nitrocellulose membrane	Amersham Biosciences
Isopropanol for molecular biology	Sigma
Kanamycin sulphate	Sigma
Leupeptin	Roche
Lipofectamine 2000	Invitrogen
L-leucine	Formedium
Luria Broth	Sigma
MEM Non-essential amino acid solution 100x	Sigma
Minimum essential medium eagle balanced salt solution	Sigma
McCoy's 5a Medium	Sigma

Nutlin-3	Sigma
Opti-MEM medium	Gibco
Pepstatin	Roche
Phenol (equilibrated), pH = 8	Sigma
Phenylmethanesulfonyl fluoride	Fluka
RNA-bee	Biogenesis
RPMI-1640 medium	Sigma
SDS (sodium dodecyl sulfate)	Fluka
Sodium bicarbonate solution	Sigma
Sodium chloride	GPR
Sodium dihydrogen phosphate dihydrate	Fluka
Sodium phosphate dibasic dihydrate	Reidel-de Haen
Sodium pyruvate	Sigma
Soybean Trypsin Inhibitor	Roche
TEMED (N,N,N',N'Tetramethylethylene-diamine)	VWR
Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol)	Calbiochem
Triton X-100	Fluka
Trypsin – EDTA solution	Sigma
Tween 20	USB
Western lightning chemiluminescence reagent plus	Perkin Elmer Life Sciences
X-beta-galactosidase (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside)	Apollo Scientific

X-alpha-galactosidase (5-bromo-4-chloro-3-indolyl α-D-galactopyranoside)	Apollo Scientific
Xylene cyanol	Sigma
Yeast nitrogen base without amino acids	Formedium
YEP broth	Formedium
YPD Agar	Formedium

Table 2.1 List of reagents.

2.2 Buffers and solutions

Electrophoresis running buffer

25mM Tris base

250mM glycine

0.1% w/v SDS

Electrophoresis transfer buffer

25mM Tris

192mM glycine

20% v/v methanol

4x protein sample buffer

0.25M Tris (pH 6.8)

8% w/v SDS

40% v/v β-mercaptoethanol

0.4% w/v bromophenol blue

10x DNA/RNA native loading buffer

50% v/v	glycerol
1mM	EDTA
0.1% w/v	bromophenol blue
0.1% w/v	xylene cyanol

5x denaturing RNA loading buffer

50% v/v	formamide
40% v/v	glycerol
0.1% w/v	bromophenol blue
0.1% w/v	xylene cyanol

Buffer E (10x)

0.18M	Na_2HPO_4
0.02M	NaH_2PO_4

RNA denaturing sample buffer

9.25 % v/v	formaldehyde
75% v/v	formamide

Sample preparation for the denaturing agarose gel electrophoresis

18µl of 10x E buffer was added to 9,2µl of the RNA denaturing sample buffer, 2µl (10µg) of RNA, and 6µl of the denaturing RNA loading buffer.

PBS/Tween

0.065M	Na_2HPO_4
0.015M	NaH_2PO_4
0.075M	NaCl
0.1% v/v	Tween 20

SLIP (Stuart Linn immuno-precipitation) buffer (per 100ml)

0.05M	HEPES pH=7.5
1% v/v	glycerol
0.1% v/v	Triton X-100
150mM	NaCl
0.05% w/v	BSA

TAE buffer (50x)

2M	Tris base
2M	glacial acetic acid
50mM	EDTA

2.3 Media

Luria broth (1L) liquid medium

25g of powdered LB dissolved in 1L of water and autoclaved.

Luria broth agar (1L)

25g powdered LB

15g agar

Water added to 1L and autoclaved.

YPDA liquid medium (1L)

30g of YEP broth dissolved in 885 ml of water, autoclaved;

Following autoclaving 100 ml of 20% w/v sterile glucose and 15 ml of sterile 0.2% w/v adenine hemisulphate salt was added.

YPDA+ liquid medium (for yIG397 FASAY yeast)

30g of YEP broth dissolved in 885 ml of water, autoclaved; following autoclaving 100 ml of 20% w/v sterile glucose and 20ml of 1% w/v adenine hemisulphate salt was added.

DO liquid media (1L)

6.9 g of yeast nitrogen base without amino acids was dissolved in 850 ml of water and autoclaved. The following supplements were dissolved in 50 ml of water:

- Leu DO liquid medium: 0.69g of –Leu DO supplement;
- Trp DO liquid medium: 0.64g of –Leu/-Leu DO supplement and 0.1g of leucine;
- Leu/Trp DO liquid medium: 0.64g of –Leu/-Leu DO supplement
- Leu/Trp/His/Ade DO (quadruple, QDO) liquid medium: 0.60g of –Leu/-Trp/-His/-Ade DO supplement,

filter sterilised and added to the yeast nitrogen base solution as required. 100 ml of 20% w/v glucose was subsequently added. All media, except of QDO, were supplemented with 15 ml of sterile 0.2% w/v adenine hemisulphate.

YPDA agar medium (1L)

70 g of YPD agar was added to 985ml, autoclaved and supplemented with 15 ml of sterile 0.2% w/v adenine hemisulphate salt.

YPDA+ agar medium (1L) (for yIG397 FASAY yeast)

70 g of YPD agar was dissolved in 985ml, autoclaved and supplemented with 20ml of 1% w/v of adenine hemisulphate salt.

DO agar media (1L)

6.9 g of yeast nitrogen base without amino acids was dissolved in 850 ml of water, 20g of agar was subsequently added and autoclaved. The following supplements were dissolved in 50 ml of water:

- Leu DO agar medium: 0.69g of –Leu DO supplement;
- Trp DO agar medium: 0.64g of –Leu/-Leu DO supplement and 0.1g of leucine;
- Leu/Trp DO agar medium: 0.64g of –Leu/-Leu DO supplement;
- Trp/His DO agar medium: 0.60g of –Leu/-Trp/-His/-Ade DO supplement and 20mg of adenine hemisulphate salt and 0.1g of leucine;
- Leu/Trp/His DO (triple, TDO) agar medium: 0.60g of –Leu/-Trp/-His/-Ade DO supplement and 20mg of adenine hemisulphate salt;
- Leu/Trp/His/Ade DO (quadruple, QDO) liquid medium: 0.60g of –Leu/-Trp/-His/-Ade DO supplement,

filter sterilised and added to the yeast nitrogen base solution as required. 100 ml of 20% w/v glucose was subsequently added. All media, except of QDO, were supplemented with 15 ml of sterile 0.2% w/v adenine hemisulphate.

–Leu/Ade (low adenine concentration) DO solid medium (1L) (for yIG397 yeast FASAY screening)

6.9 g of yeast nitrogen base without amino acids was dissolved in 850 ml of water, then 20g of agar was added and autoclaved. 0.60g of –Leu/-Trp/-His/-Ade DO was combined with 4mg of adenine hemisulphate salt, 20mg of histidine, 20mg tryptophan and dissolved in 50 ml of water followed by filter sterilization. The amino-acid solution was then added to the autoclaved yeast nitrogen base - agar solution. 100 ml of 20% w/v glucose, and water was added to a final volume of 1L.

Yeast freezing medium

30g of YEP broth was dissolved in 500 ml of water and autoclaved. 100 ml of 20% w/v sterile glucose, 15 ml of sterile 0.2% w/v adenine hemisulphate salt, 250ml of sterile glycerol and water was added to a final volume of 1L.

Sterile 20% w/v glucose water solution (1L)

200g of D-(+)-glucose dissolved in 1L of water and autoclaved.

2.4 Enzymes

Enzyme	Manufacturer
BamH I	NEB
Bgl II	NEB
Cla I	NEB
EcoR I	NEB
EcoR V	NEB

Hind III	NEB
Nco I	NEB
Nhe I	NEB
Not I	NEB
Sac I	NEB
Xho I	NEB
Xba I	NEB
Taq DNA polymerase	Eppendorf
Pfx DNA polymerase	Invitrogen
Advantage DNA polymerase	Clontech
Lyticase	Sigma
MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase	Clontech
Antarctic phosphatase	NEB

Table 2.2 List of enzymes

2.5 Primers

Name of the primer	**Sequence (5' to 3')** N=A, G,C, or T; V=A,G, or C
CDS III primer	ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$VN,
BD SMART III primer	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG
cDNA 5' PCR Primer	TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG

cDNA 3' PCR Primer	GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA
5' AD LD-Insert Screening Amplimer:	CTATTCGATGATGAAGATACCCCACCAAACCCAAAAAAAG AG
3' AD LD-Insert Screening Amplimer	AGTGAACTTGCGGGGTTTTTCAGTATGTACGAT
NME2 for pFLAG-CMV-6b	CTCGAATTCCCCGGACCATGGCCAACCTGG
NME2 rev pFLAG-CMV-6b	GCTGTTGGATCCACCTCTTATTCATAGACCC
NME1 for pFLAG-CMV-6b	GATGAATTCGGACCATGGTGCTACTGTCTACTTTAG
NME1 rev pFLAG-CMV-6b	GTGGGATCCCCTCCTGTCATTCATAGATCC
NME2 H118F pCEP for	GAGCGGCCGCGGACCATGGCCAACCTGGAGCGCACCTTCA T
NME2 H118F pCEP rev	GAGGATCCTTATTCATAGACCCAGTCATGAGCACAAGACC TTGTAGTCAACCAGTTCTTCAGGCTTAAACCATAGGCTGAT TTCTTTTTCAGCACTTTTTACTGAATCACTGCCAAAATG
NME2 K12Q pCEP for	GAGCGGCCGCGGACCATGGCCAACCTGGAGCGCACCTTCA TCGCCATCCAGC
NME2 K12Q pCEP for	GAGCGGCCGCGGACCATGGCCAACCTGGAGCGCACCTTCA TCGCCATCCAGC
NME2 K12Q pCEP rev	GCTGTTGGATCCACCTCTTATTCATAGACCC
P3 FASAY	CCTTGCCGTCCCAAGCAATGGATGAT
P4 FASAY	ACCCTTTTTGGACTTCAGGTGGCTGGAGT

Table 2.3 List of primers

2.6 Vectors

Name of the vector	**Source**
pGBKT7 DNA-BD cloning vector	Clontech (Matchmaker kit)
pGADT7-Rec AD cloning vector (SmaI linearized)	Clontech (Matchmaker kit)
pGBT9 transformation efficiency control plasmid	Clontech (Matchmaker kit)
pGBKT7-p53 control vector	Clontech (Matchmaker kit)
pGBKT7-Lam control vector	Clontech (Matchmaker kit)
pCR.2.1 vector	Invitrogen
pFLAG-CMV-6b (NME1 and NME2)	Sigma
pCEP4 (NME1 and NME2 wt and mutants)	Invitrogen
pRDI-22	Obtained from prof. Richard Iggo, (Waridel et al., 1997)
pCEP4-p53	Obtained from dr Dale Haines (Boyd et al., 2000)
pCMV-neo-bam-hMDM2	Obtained from prof. Bert Vogelstein (Oliner et al., 1992)

Table 2.4 List of vectors

2.7 Antibodies

Antibody	**Manufacturer**
Anti-p53 (ab-6) DO-1 mouse monoclonal, primary	Calbiochem
Anti-MDM2 (ab-1) mouse monoclonal, primary	Calbiochem

Anti-actin c-2 mouse monoclonal, primary	Santa Cruz Biotechnology
Anti-p21$^{CIP1/WAF1}$ F-5 mouse monoclonal, primary	Santa Cruz Biotechnology
Anti-β Galactosidase mouse monoclonal, primary	Calbiochem
NME2 L-15 gout polyclonal, primary	Santa Cruz Biotechnology
NME1/2 rabbit polyclonal, primary	Abcam
Bax (ab-1), rabbit polyclonal, primary	Calbiochem
Anti-mouse antibody, HRP-conjugated, secondary	Amersham Biosciences
Anti-mouse antibody, HRP-conjugated, secondary	Amersham Biosciences
Anti-goat antibody, HRP-conjugated, secondary	Jackson

Table 2.5 List of antibodies

2.8 Short interfering RNA (siRNA)

Short interring RNA (siRNA) oligonucleotides were designed by Dr Mark T Boyd, Dharmacon company or others (where cited).

Scrambled

Target sequence: 5'- AAGGACGCAUCCUUCUUAAUU –3'

p53 (Martinez et al., 2002)

Target sequence: 5'- AAGCAUGAACCGGAGGCCCAU -3'

MDM2

Target sequence: 5'- AAGCCACAAAUCUGAUAGUAU -3'

NME2 oligo 8

Target sequence: 5'- AAGGCGAGAUCAUCAAGCGCUU -3'

2.9 Cell culture

Tissue culture was performed in a tissue culture hood using sterile technique. Cells were grown in humidified incubators at 37^0C with 5% CO_2. Media, providing nutrients for optimal growth for individual cell lines, are described below.

H1299 cells (non-small cell lung carcinoma cells) were grown in the RPMI 1640 medium supplemented with 10% FBS.

MCF-7 (breast carcinoma) cell line was maintained in the DMEM Hepes modified supplement medium, supplemented with 10% FBS and 2mM L-glutamine.

BJ fibroblasts (human dermal fibroblasts) were maintained in MEM Eagles balanced salt solution supplemented with 10% FBS, 2mM L-glutamine, 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids and 1mM sodium pyruvate.

117, ACHN, A498 (RCC) cell lines were maintained in Minimum Essential Medium Eagle supplemented with 10% FBS, 2mM L-glutamine, 1.5g/L sodium bicarbonate, 0.1mM non essential amino acids, and 1mM sodium pyruvate.

Caki-2 (RCC) cell line was maintained in the McCoy's 5a medium supplemented with 10% FBS, 1.5mM L-glutamine and 2.2g/l sodium bicarbonate.

786-O (RCC) cell line was maintained in the RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4.5g/l glucose and 1.5g/l sodium bicarbonate.

2.10 RNA extraction, synthesis and amplification of cDNA

Total RNA extraction

Cells were lysed with 15ml of RNA-bee. 0.2 volumes of chloroform was added, shaken vigorously for 15 seconds and the lysate was put on ice for 15minutes. The

lysate was then transferred into the screwcap microcentrifuge tube and centrifuged for 15 minutes at 4°C at 14.200g. The aqueous phase was removed and an equal volume of isopropanol was added, placed on ice for 15 minutes and centrifuged for 15 minutes at 4°C at 14.200g; isopropanol was removed, the pellet resuspended in 70% v/v ethanol and placed O/N at -20°C. The precipitate was centrifuged for 15 minutes at 4°C at 14.200g, ethanol was removed, the pellet was air-dried and dissolved in 250µl of water.

The OD at 260 and 280nm was measured to evaluate purity and yield of RNA.

mRNA purification (RNeasy, Qiagen kit)

mRNA was purified using Qiagen columns following the manufacturer's instructions. Typically, 250 µg of total RNA was loaded onto each column eluted twice with 20µl of OEB buffer followed by measurement of OD at 260 and 280nm to determine yield and purity of the sample.

Native agarose gel electrophoresis

Typically, 0.5µg of mRNA, 1µg of total RNA, and 1µg of total control RNA was run on a 1.2% w/v agarose gel containing ethidium bromide (60µg/ml), using 1x TAE buffer. The gel was initially run at 100mA for the first 10 minutes and then at 20mA for next 7 hours, with buffer circulation in the tank to prevent forming of the pH gradient across the tank, as basic pH causes auto-degradation of RNA. Following electrophoresis, the gel was distained for 30 minutes in water to decrease background fluorescence, RNA was visualized using transluminometer and photographed using a Kodak EDAS system.

Denaturing agarose gel electrophoresis (per 100ml gel)

1.2g of agarose and 10ml buffer E were added to 73.3ml of water and boiled in the microwave machine until agarose was dissolved. Upon cooling to < 55°C, 16.7ml of 37% formaldehyde was added, mixed and the gel was poured into a previously prepaired tray. RNA samples were heated at 65°C for 5 min. placed on ice for another 5 minutes and briefly centrifuged. Then, RNA samples were loaded on the gel and separated at 100mA for the first 10 minutes and at 20mA for the next 7h. The 1x E electrophoretic buffer was constantly circulated using a peristaltic pomp in order to prevent formation of pH gradient across the tank. Subsequently, the gel was distained for 30 minutes in water and photographed using Kodak Edas system.

cDNA synthesis

For the first strand cDNA synthesis, 1μl of mRNA (1μg/μl) was used, then 2μl of this was used for cDNA amplification and purification. All procedures were performed using Matchmaker library construction and screening kit according to the BD Biosciences Clontech protocol #PT3529-1, version #PR32047.

2.11 The yeast two-hybrid system

The yeast two-hybrid system, used in this study, is based on two domains of the GAL4 transcription factor which can not interact with each other (thus, to reconstitute the functional Gal4 transcription factor) unless they are connected by the bait and library proteins (Fields and Song, 1989). pGADT7-Rec plasmid encodes a fusion of the GAL4 activation domain and the library protein and pGBKT7 produces the bait protein fused to the DNA binding domain of the GAL4 transcription factor. If the library and bait proteins interact, the reconstituted GAL4 is able to activate

reporter genes and the yeast will grow on selective media (Figure 2.1). Yeast two-hybrid screen is a high throughput approach allowing efficient screening of the library for proteins that interact with the bait. The screen is preceded by numerous steps of preparation and optimization, which are described below.

Test for the DNA-BD-MDM2 fusion toxicity for the Y187 yeast strain

The DNA-BD-bait protein may be toxic to the yeast cells which could affect quality of the screen or assay by decreasing the rate of cell growth and division. To test for toxicity of the fusion protein, yeast cells transformed with the bait vector were grown in the appropriate DO liquid medium and the effect of the fusion in the yeast cells was determined by measurement of the culture density as described below.

50ml of the -Trp DO liquid medium was inoculated with one large colony from the -Trp DO agar plate and grown for 16-24h in the shaking incubator at 30°C. If after 24h the culture does not reach $OD_{600} = 0.8$, it means that the fusion protein is toxic for yeast.

Preparation of competent yeast cells

Preparation of competent yeast cells is a key procedure in achieving high transformation efficiency which is crucial for construction of a high quality cDNA library. A large number of individual clones is important as this gives a chance for less abundant cDNA species to be present in the library. The large number of individual clones in the library is dependent on high transformation efficiency. To achieve this, the competent yeast cells must be prepared carefully.

Preparation of competent yeast cells was carried kit according to the BD Biosciences Clontech protocol #PT3529-1, version #PR32047.

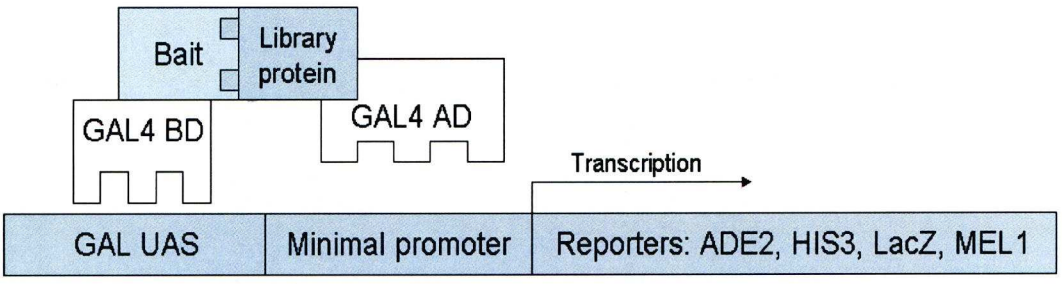


Figure 2.1 **Principle of the yeast-two-hybrid system.** The Diagram illustrates the molecular mechanism of the yeast two-hybrid-GAL4 system. Interaction between the bait and the library protein fused to the binding and activation domains, respectively, lead to activation of reporter genes.

Library construction

A cDNA library constructed in yeast is a pool of yeast cells transformed with vectors containing cDNA from defined sources, such as tissues or cell lines. In case of this work, cDNA for construction of the library was obtained by reverse transcribing mRNA derived from a cancer cell line. The procedure of cDNA preparation for construction of the library was described before (2.10.) The procedure of library construction was performed according to the BD Biosciences Clontech protocol #PT3529-1, version #PR32047.

The pool of transformed yeast cells constituting the library was mixed thoroughly, aliquoted (1ml) and stored in -80°C. The following day one library vial was thawed; dilutions were performed and plated on 15cm –Leu DO agar plates to estimate the library titre.

Mating

Yeast mating is a process based on fusion of two compatible, haploid yeast cells which subsequently form a diploid cell. This corresponds to fertilization because haploid yeast cells act as gametes and form a zygote. The process of mating is used for combining yeast strains transformed with library and bait plasmids growing on single DO media. After mating, yeast are transferred on multiple DO media to select for successfully mated cells (double DO) and yeast bearing plasmids producing weakly or strongly interacting fusion proteins (triple and quadruple DO media, respectively). The procedure of yeast mating was carried out according to the Clontech protocol #PT3529-1, version #PR32047. Following mating, cells were selected for yeast two-hybrid positives on 15cm –Trp/-Leu/-Ade/-His DO agar plates and incubated upside down for 8 days at 30°C.Subsequently, yeast colonies were

then picked and transferred onto fresh –Leu/-Trp/-His/-Ade DO agar plates using sterile toothpicks and incubated upside down for 3 days at 30°C. Out of these, the largest clones were picked and inoculated onto –Leu/-Trp/-His/-Ade/X-α-gal DO agar Petri dishes in order to estimate activation of the MEL1 reporter gene. The strongest positives were subjected to PCR for further restriction digest (to discriminate between identical clones) and sequencing of clones that exhibit unique fingerprint.

Positive yeast two-hybrid colonies were screened for the presence of inserts by PCR.

The PCR reaction was composed of 2.5µl of 10x polymerase buffer, 0.5µl of 40mM dNTP mix, 1µl of 5', 3' screening amplimer mix (5pmol/µl each), 0.25µl (1.25U) of the Taq polymerase and 20.75µl of water to total volume of 25µl. A tiny fraction of colony was picked using a sterile toothpick and dipped into the reaction mixture prior to start of the reaction.

Cycling was programmed as follows:

Initial denaturation for 3 minutes at 95°C was followed by 30 three-step cycles of denaturation for 30 seconds at 95°C, 30 seconds of annealing at 68°C and extension at 72°C for 4 minutes.

2.12 The FASAY (Functional Analysis of Separated Allele in Yeast)

p53 is mutated in at least 50% of human cancers and its inactivation is generally essential in the process of tumourigenesis (Vousden and Lu, 2002). In some cancers, mutation of p53 is associated with progression of the disease (reviewed in Boyd, 2008). Therefore, highly sensitive (because some tumour samples are very

heterogeneous) screening strategies for p53 mutation are important for administration of the appropriate treatment.

One of such screening techniques is yeast-based FASAY which allows searching for relatively rare mutant p53 alleles in heterogeneous samples. The technique is based on recombination of the p53 cDNA with the vector (pRDI-22 vector used here) carrying the Leu2 gene which allows the yeast cells to grow on –Leu DO media. Wt p53, recombined with the pRDI-22 vector, activates expression of the Ade2 gene encoded by another vector, pLS210, stably integrated with the genome of the yIG397 yeast strain, used for FASAY. Yeast cells, expressing the Ade2 gene are able to form large white colonies. Mutant p53 is unable to induce expression of the Ade2 gene and cells that carry the pRDI-22 vector recombined with mt p53, form small and red colonies (resulting from accumulation of a red pigment, ribosylaminoimidazole (Smirnov et al., 1967), since there is too little adenine in the medium to allow robust growth. The distinct morphology of colonies carrying mt p53 allows efficient screening and straightforward identification of the mutation (Camplejohn and Rutherford, 2001).

Total RNA was obtained from the tumour sample and 1-5μg of RNA was reverse transcribed using oligo-dT and the M-MuLV reverse trascriptase in the total reaction volume of 20μl incubated for 1h at 42°C. The PCR reaction was then performed with 2μl of cDNA, FASAY P3 and P4 primers and the Phusion polymerase in the total volume of 50μl. The cycling was programmed as follows: 30sec. of initial denaturation at 98°C was followed by 35 cycles of 10 sec. denaturation at 98°C, 30 sec. annealing at 58°C and 40 sec. extension at 72°C. The PCR product was purified from the 1.2% agarose gel. The pRDI-22 was sequentially cut with HindIII and StuI (digestion of the vector greatly increases recombination efficiency) and 250ng was

cotransformed with 125ng of the PCR product into the competent yIG397 yeast cells (prepared and transformed using the Li/Ac method (according to the Clontech #PT3529-1 manual). Subsequently, yeast were plated on solid FASAY screening media and incubated up side down at 31°C for 3-4 days and then transferred to 4°C for the red colour to develop. Red and white colonies were scored and 5 red colonies for each sample were subject to PCR using the same protocol as for amplification of the p53 cDNA. The PCR products were subsequently separated on the 1% agarose gel, out of which two to four (for each tumour or cell line) were purified and sent for sequencing.

2.13 Plasmid production

Small scale plasmid DNA extraction from E.coli

For screening and yeast transformation purposes small scale plasmid DNA extraction was performed using Quiagen miniprep kit according to the manufacturer's protocol.

Large scale plasmid DNA extraction from E.coli

For the purpose of transfection experiments, to obtain high quality DNA, large scale plasmid DNA extraction was performed using Quiagen megaprep kit according to the manufacturer's protocol.

2.14 Molecular cloning

Molecular cloning is a process of incorporation of the usually defined fragment of DNA into the vector. Various different cDNAs were cloned in this study to obtain constructs suitable for protein expression in yeast and mammalian systems.

Cloning of NME2 into pFLAG-CMV-6b vector

NME2 cDNA was amplified by PCR reaction composed as follows:

5µl of 10X *Pfx* amplification buffer was mixed with 0.5µl of 40mM dNTP mix, 1µl primer mix (NME2 for pFLAG-CMV-6b and NME2 rev pFLAG-CMV-6b, 5pmol/µl each), 1µl of placental cDNA, 0.7µl Pfx DNA polymerase (2.5U/µl) and 41.8µl of water up to total volume of 50µl in a 200µl PCR tube. The sample was subject to initial denaturation for 2 minutes at 95°C, followed by 10 three-step cycles of denaturation for 30 seconds at 95°C, 30 seconds of annealing at 60°C and extension at 68°C for 45 seconds. Then 1µl of Taq polymerase (5U/µl) and 2µl of dATP (10mM) was added to the reaction and incubated for 20 minutes at 72°C. The PCR product was then purified using the Geneclean kit and eluted in 20µl and 6µl of the amplified NME2 cDNA was mixed with 1µl of T4 ligase, 1µl of T4 ligase buffer and 2µl (25ng/µl) of the pCR2.1 vector and the ligation reaction was carried out O/N at 14°C.

The following day, the ligation reaction was used to transform the Top-10 E. coli bacteria according to the manufacturer's protocol follwed by spreading on the ampicillin/X-gal/agar 10cm Petri plates and incubation upside down O/N at 37°C to allow selection of transformants.

The pCR2.1 vector allows blue/white colony screening of transformants. The β-galactosidase encoded by the lacZ in the pCR2.1 plasmid, digests X-gal present in the media to the blue product, hence the colony appears blue. The multicloning site is present within the lacZ gene; insertion causes inactivation of the gene which is not expressed in such a case and the colony appears white. Blue colonies are formed by bacteria carrying a pCR2.1 vector without the insert; these colonies should not be propagated.

A white colony was picked and propagated O/N in 3ml of LB liquid medium at 37°C followed by Qiagen miniprep plasmid extraction according to the manufacturer's protocol. DNA was eluted in 30µl of water, 2µg was sent to the MWG company for sequencing and the sequence was subsequently compared with the reference sequence from the NCBI database using BLAST two sequence alignment in order to ensure that no mutations were introduced by means of PCR.

Subsequently, 5µg of the plasmid DNA (pCR2.1-NME2) was digested with EcoRI and BamHI restriction enzyme according to the manufacturer's protocol and 5µg of the plasmid pFLAG-CMV-6b was digested with EcoRI and BamHI restriction enzyme and dephosphorylated by adding 2µl of Antarctic phosphatase and an appropriate volume of 10x buffer followed by incubation for 20 minutes at 37°C. The samples were separated on a 1.5% agarose gel and the band corresponding to the size of NME2 (~500bp) and the pFLAG-CMV-6b backbone were extracted and purified using the Geneclean (Qbiogene) reagents and protocol.

An O/N ligation reaction of the NME2 - pFLAG-CMV-6b was setup using 50ng of the vector and as much of the insert as possible. The ligation samples were used to transform XL1-blue E. coli bacterial strain (Stratagene) according to the manufacturer's protocol followed by spreading of the bacterial suspension on ampicillin agar Petri dishes and O/N incubation at 37°C. After 16h, a colony was picked and propagated O/N in 3ml of LB liquid medium at 37°C and was then subject to the Qiagen miniprep plasmid DNA extraction . DNA was eluted in 30µl of water.. 5µl was digested with of either EcoRI or BamHI enzymes and presence of the insert confirmed following 1,5% agarose gel electrophoresis.

100µl of the 3ml O/N culture was used to inoculate a 500ml overnight culture. A megaprep plasmid preparation protocol was followed to obtain high quality DNA.

Cloning of NME1 into pFLAG-CMV-6b vector

NME1 was cloned essentially as described above for NME2. The PCR primers used for amplification of NME1 cDNA were:

NME1 for pFLAG-CMV-6b and NME1 rev pFLAG-CMV-6b

Subcloning of NME2 into pCEP vector

5μg of the pFLAG-CMV-6b- NME2 and the pCEP-empty vector were digested using HindIII and BamHI enzymes, the pCEP vector was then dephosphorylated as described before. The samples were then separated on a 1.5% agarose gel, the pCEP vector band and the 500bp band of the NME2 cDNA were extracted and purified using the Geneclean kit according to the manufacturer's protocol.

An O/N ligation reaction of the NME2 pCEP4, transformation into XL1-blue cells and a megaprep plasmid preparation were set up, as described previously (2.10).

Subcloning of NME1 into pCEP vector

NME1 was sub-cloned into pCEP4 vector as described above, but the NotI endonuclease was used instead of HindIII for the 5' end of the NME1 sequence.

Generation of NME1 and NME2 chimeric constructs

Both NME1 and NME2 cDNAs were excised from a pCEP vector using NotI and BamHI (NME1) or HindIII and BamHI (NME2) and were then digested with an enzyme PflM1 recognising a conserved restriction site at position 227 (exactly in the middle of the coding sequence). Subsequently, the N-terminal part of NME1 was ligated with the C-terminal part of NME2 and the pCEP vector, previously linearized using NotI and BamHI restriction endonucleases. Additionally, the C-terminal part of

NME1 was ligated with the N-terminal part of NME2 and the pCEP vector, previously linearized using HindIII and BamHI restriction endonucleases. The O/N ligation reaction mixes were then used for transformation into XL1-blue cells and a megaprep plasmid preparationwas performed as described previously (2.10).

2.15 DNA sequencing

Sequencing is a technique allowing determination of DNA sequence. In the case of this project, sequencing of yeast library cDNAs obtained from the yeast two-hybrid positive clones, was performed as follows.

2µl of the 10x buffer was mixed with 4µl Terminator Ready Reaction Mix, 4µl primer (1.6 pmoles), 2µl DNA and 8µl H_20 in a well of a 96-well PCR plate. The sample was subject to 25 three-step cycles of denaturation for 10 seconds at 96°C, 5 seconds of annealing at 50°C, extension at 60°C for 4 minutes and cooled down to 4°C afterwards until switched off. Then, the precipitation solution, consisting of 3µl of 3M Sodium acetate (NAOAc)-pH=4.6, 62.5µl of ethanol and 14.5µl of deionised water was added to each sample, the plate was vortexed and left at RT for 15 minutes to precipitate DNA. The samples were centrifuged at 4,000 rpm for 30 minutes, the supernatant was discarded, the plate was centrifuged upside down for 1 minute at 50 x g. 150µl of 70% ethanol was subsequently added; the plate was sealed and inverted to mix, then centrifuged for 10 minutes at 4,000 rpm. and the supernatant was discarded. The plate was then inverted and centrifuged upside down for 1 minute at 50 x g to dry the sample. The sample was resuspended in 20µl of formamide and vortexed. Formamide was also put into any unused wells to avoid damage to the sequencer. The samples were then subjected to sequencing on ABI3100.

2.16 Protein analysis by SDS PAGE and western blotting

SDS-PAGE (polyacrylamide gel electrophoresis) is a process of protein separation according to their molecular mass which is based on the migration of protein molecules in the electric field. Proteins are evenly denatured by SDS, an anionic detergent, which also gives the protein a negative charge that corresponds to the size of the protein.

SDS-PAGE, preceded by protein sample preparation procedure is described below.

Protein samples were prepared as follows:

Cell pellets obtained by centrifugation of trypsinised cells at 300g were lysed with SLIP buffer containing protease inhibitors (PIs) – aprotinin, leupeptin, pepstatin, STI (soybean trypsin inhibitor) from 1,000x concentrated stock and PMSF prepared fresh as 100x concentrated (0.0174g/ml) in ethanol. Lysis was carried out for 15 minutes on ice followed by centrifugation for 10 minutes at 4°C at 16.100g.

In the meantime the spectrophotometer was calibrated for the Bradford protein assay: serial dilutions (20; 10; 5; 2,5; 1.25; 0.625 and 0.3125mg/ml) of BSA in SLIP+PIs buffer were performed and 2µl of each dilution was added to 1ml of bio-rad protein assay reagent, mixed and used to calibrate the spectrophotometer. Then, 2µl of each sample was mixed with 1ml of 1x bio-rad protein assay reagent and concentration of the protein in the sample was read using the spectrofotometer.

Typically, 50µg of the protein was mixed with 1x, 2x, and 4x sample buffer to obtain a final concentration of 1x sample buffer and 50µg of protein in the sample mix.

SDS polyacrylamide separating gel was prepared as follows

	6%	7,5%	10%	12%	15%
Water	5.8 ml	5.42ml	4.8ml	4.3ml	3.55ml
Acrylamide/bisacrylamide mix	1.5 ml	1.87ml	2.5ml	3ml	3.75ml
1.5M Tris pH=8.8	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% w/v SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% w/v APS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
TEMED	0.008ml	0.008ml	0.008ml	0.008ml	0.008ml

Table 2.6 List of substrates for a polyacrylamide gel of given percentage.

SDS polyacrylamide stacking gel

Water	7.225 ml
Acrylamide/bisacrylamide mix	1.275 ml
1M Tris pH=6.8	1.25ml
10% w/v SDS	0.1ml
10% w/v APS	0.1ml
TEMED	0.01ml

Table 2.7 List of substrates for stacking a polyacrylamide gel.

A 0.75mm thick separating gel was poured and overlaid with 1ml of water. After 30 minutes water was removed, the stacking gel was poured on the top of the separating gel and the 10-well comb was immediately inserted between the plates into the

stacking gel. The gel was allowed to polymerase for one hour prior to use. Typically, electrophoresis was carried out at 200mV. The plates were subsequently disassembled and the gel was placed on a Hybond ECL nitrocellulose membrane (pre-soaked in the transfer buffer), the gel and the membrane were placed between two 3mm Whatman paper sheets and two sponges; air bubbles were carefully removed from this "sandwich". Then the "sandwich" was placed into the tank next to the frozen cooling block, filled with the transfer buffer and transferred for 1 hour at 100mV. The membrane was removed, stained with ponceau S for 1min, excess dye washed away with water and the membrane was cut into regular strips according to the molecular mass marker bands corresponding to the MW of proteins of interest. The membranes were distained in PBS/Tween and transferred into 5% w/v blocking milk dissolved in PBS/Tween for at least 1 hour to block places on the membrane that could unspecifically bind antibodies, thus increasing the background.

Immunoblotting

Immunoblotting is a technique based on a specific recognition of the epitope by the antibody. Antibodies, which allow specific recognition of a given amino acid sequence of the protein of interest, are commercially available and were used in this study (Table 2.5). Procedure of protein detection with specific antibodies is described below. Membranes prepaired as described above, were placed in 5% blocking solution with primary antibody and agitated for 1 hour at room temperature. The primary antibody solution was then removed and the membrane washed with PBS/Tween for three times for every 10 minutes. Secondary antibody diluted (Amersham Biosciences anti mouse 1:2500, anti rabbit 1:4000, Jackson anti-goat 1:20,000) in 5% blocking solution was added to the membranes and incubated for 1

hour at room temperature. The secondary antibody solution was then removed and the blot was re-washed as above.

Detection of western blot signals

The secondary antibody is conjugated to an enzyme, peroxidase which is capable of catalysing reaction resulting in production of light. It recognizes the Fc fragment of the primary antibody. The western blot was developed using western lightning chemiluminescence reagent plus. Equal volumes of the enhanced luminal and oxidising reagents were combined and vortexed. The solution was subsequently applied to the membrane and incubated for 1 min. The membrane was then dried gently on a tissue and placed between two layers of clingfilm. Luminescence was detected on a Kodak Image Station 4000mm and saved as .jpg files.

2.17 Dual luciferase reporter assay (Promega)

p53 activity can be studied using luciferase reporter system. The pp53-TA-Luc vector, uesd here, contains a firefly (*Photuris lucicrescens,* a beetle producing luminescence) luciferase gene. Luciferase is an enzyme, capable of emitting light which can be detected. Expression of luciferase from the pp53-TA-Luc vector is controlled by the herpes simplex virus thymidine kinase promoter and a p53 response element composed of the part of the putative replication origin of the human ribosomal gene cluster and a p53-binding consensus sequence.

The second reporter vector, pRL-TK, was used in the assay. It encodes a luciferase gene from *Renilla reniformis* (a polyp forming anthozoan animal living in the sea), controlled by the herpes simplex virus thymidine kinase promoter. Renilla luciferase

activity is independent of p53 and is detected independently of the firefly luciferase used in the same assay, therefore it serves as a internal control.

The assay was performed according to the manufacturer's manual using the GloMax 20/20 Luminometer.

2.18 Transfection of plasmid DNA into cells

Transient transfection procedure aims at introduction of DNA into cells. For this purpose the cells were seeded in 6-well plates at density leading to subconfluency after 48h of incubation at 37°C. 24 hours after seeding the cells were transfected with Gene juice [μl] : vector DNA [μg] at a ratio of 3: 1 ([2.5:1] in BJ fibroblests) according to the manufacturer's protocol. Typically, of 1.2μg of the pp53-TA-Luc vector expressing luciferase and 0.2μg of the pRL-TK vector expressing renilla (when transfection was performed for the purpose of a subsequent luciferase assay), 0.3μg of the pSUPER vector expressing GFP as internal controls, 0.02μg pCEP-p53 vector expressing p53 (0.04μg in BJ fibriblasts), 0.06μg of pCMV-neo-bam-MDM2 vector expressing MDM2 (0.04μg in BJ fibroblasts) and 3μg of pCEP-NME expressing NME1 or NME2 was transfected per one well of the six well plate. Where indicated, larger amounts of MDM2 and p53 vectors were used.

2.19 Transfection of siRNA into cells

Introduction of specific siRNAs (small interfering RNAs) into cells results in a transient "knockdown" of gene expression. siRNAs target mRNA of the particular gene for degradation, which results in downregulation of its expression. For this purpose cells were seeded in 6-well plates or 10cm dishes at density leading to

subconfluency after 72h of incubation. 24 hours after seeding the cells were transfected with Lipofectamine 2000 (4µl per each well). 80 pmoles of siRNA (40nM concentration) was used for each well of the six-well plate. The procedure was carried out according to the manufacturer's protocol.

6 hours after transfection (depending on the purpose of the experiment and the cell line), the media were replaced to avoid the cell stress and death caused by toxic properties of the transfection reagent. 48 hours after transfection the cells were washed with PBS, three wells of each plate were harvested for further applications by adding 0.5ml of trypsin-EDTA solution into each well of the 6-well plate. After cell detachment, trypsin was neutralized using media with 10% FBS and the cell suspension was collected. Depending on the subsequent application, the cell were counted (using the Beckman coulter counter) and seeded or centrifuged for 5 min. at 300g. After centrifugation the pellet was washed with PBS, centrifuged again and frozen for protein analysis by western blotting.

2.20 Boyden chamber motility assay

Boyden chamber motility assay measures the intrinsic ability of cells to migrate. The bottom of the chamber, used in this experiment, contains a porous membrane (pore size 8µm) which serves as a barrier for the non-motile cells. Motile cells, however, actively move through the pores to the other side of the membrane and can be quantified upon fixing and staining.

Optimization of seeding density

600µl of media was added to the wells of the 24-well companion plate and the Boyden chamber inserts were placed into the wells. Cells were harvested as

described in Section 2.18, and counted. 5,000, 10,000, 15,000 and 50,000 cells was seeded in the volume of 300µl into the Boyden chambers inserted into the companion plate and placed immediately in the incubator for 18h. Then, the cells were carefully wiped of the inner side of the chamber using cotton swabs, washed in PBS, fixed and stained using the REASTAIN kit according to the manufacturer's protocol. Subsequently, the membrane was cut off the chamber, placed on the microscopic slide on a drop of the DPX mounting solution, overlaid with another drop of DPX and covered with a cover slip.

The cells were counted on the microscope; a grid was drawn on the cover slip if necessary. The seeding density yielding approx. 1,000-10,000 cells that migrated through the membrane of the chamber was used in subsequent experiments.

Motility assay

The cells were grown in culture for at least 48h in triplicates, harvested, counted and the experiment was carried out essentially as described above.

2.21 Characterization of the cell-based RCC tumour progression model and cell lines spontaneously over-expressing p53 and MDM2

Several years ago, members of our research group proposed and attempted to generate a cellular model of RCC tumour progression. It was based on the observation, that high levels of p53 correlate with intermediate prognosis and upregulation of both p53 and MDM2 is correlated with poor outcome in RCC patients (Haitel et al., 2000). Preliminary experiments have shown that cells failed to express high levels of wt p53. It has therefore been concluded, that upregulated p53

in RCC may be mutated. Preliminary results have also confirmed the observation made by Haitel et al. in a clinical study, that MDM2 is rarely upregulated in tumour samples harbouring low levels of p53; no cell lines overexpressing MDM2 alone have been generated. This finding also suggests that RCC cells do not tolerate high levels of MDM2.

To test if cell lines overexpressing mutant p53 (it has been assumed that the levels of p53 in RCC are high because of the mutation) tolerate high levels of MDM2, a series of stabile transfections have been performed. For this experiment, UOK 117 RCC cell line, expressing low levels of endogenous wt p53 and MDM2 proteins has been selected. Cells have first been transfected with DNp53 (dominant negative mutant of p53 R175H), pCEP vector as a control and subsequently selected for stabile transfectants. Several clones have been obtained and these cells expressed high levels of DNp53. Subsequently, to test if expression of the mutated p53 allows the cells to over-express MDM2 (which would reflect the situation in RCC described by Heitel et al.) the DNp53, as well as pCEP vector control clones have been subject to second round of transfections with either MDM2 or pCMV-neo-bam vector as a control and selected for stabile transfectants. Clones expressing high levels of MDM2 and DNp53 have now been identified. Interestingly, after second round of cloning, some clones overexpressing MDM2 could be found on plates where MDM2 has been transfected in to the pCEP control cells. However, unexpectedly, also some clones from double empty vector control transfections, spontaneously started to express MDM2. Most interestingly, these clones also over-expressed p53 which further supported the notion, that high levels of MDM2 are dependent on upregulation of (as it was initially presumed, mutated) p53.

3 Results

3.1 Identification of MDM2 binding proteins in RCC cells using a yeast two-hybrid system

3.1.1 Introduction to the yeast-two hybrid system

Over-expression of MDM2 in RCC correlates with poor outcome (see Section 1.4) and the basis of the activity of MDM2 which promotes tumour aggressiveness is unknown. The aim of this project was the elucidation of the MDM2-mediated mechanism responsible for promoting tumour progression. Since all of the known functions of MDM2 are mediated via protein-protein interactions, it is likely that MDM2 contributes to poor outcome in RCC patients by binding to other proteins. It was hypothesized that MDM2 binding resulting in altered function/s of the target protein/s would contribute to aggressiveness of renal tumours. Therefore, this project aimed at the identification of proteins interacting with MDM2 in RCC cells. To achieve this, a cDNA library was constructed from an RCC cell line and transformed into yeast and a yeast two-hybrid screen for MDM2 interacting proteins was performed (see Section 3.1.3).

The yeast two-hybrid system allows detection of interactions between two proteins that are fused to either the DNA binding or activation domain of the Gal4 transcription factor (see Diagram 2.1). Interaction is manifested by activation of marker genes that enable auxotrophic yeast cells to grow on selective media. The yeast two-hybrid method, unlike various *in vitro* techniques, provides the opportunity of investigating proteins synthesized and folded under natural conditions in a eukaryotic cell.

Overview of the strategy for the yeast two hybrid screen

In order to perform the screen for the MDM2 binding proteins, a construct expressing MDM2 as a fusion with the Gal4 DNA binding domain was transformed into an appropriate yeast strain (a mating partner for the strain to be used to express the cDNA library). Then, the cDNA library had to be constructed in the appropriate yeast cells using mRNA from the 786-0 RCC cell line (for a description of the reasons for choosing this line see 4.1). Subsequently, the MDM2- and library-expressing yeast strains were mated and selected for clones expressing the library protein interacting with MDM2. cDNAs encoding the putative MDM2 interacting proteins selected in the screen were then identified by sequencing.

3.1.2 The yeast two hybrid screen

3.1.2.1 Construction of the cDNA library

Preparation of mRNA for cDNA synthesis

To construct the cDNA library, total RNA was extracted from 786-0 cells. Based on absorbance measurements at 260nm and 280nm, the calculated concentration of the sample was 6.24µg/µl, the total yield was 1.56mg with an $A_{260/280}$ =2.00. Contamination with DNA ($A_{260/280}$ ratio of pure DNA is expected to be around 1.8), proteins or phenol decreases the $A_{260/280}$ ratio of the RNA sample as these substances absorb light of longer wavelength than RNA. Therefore, the RNA sample is regarded as pure, when the $A_{260/280}$ ratio is between 2 and 2.1. The obtained results then suggest that the RNA was not contaminated with substantial amounts of protein, phenol or DNA (Wilfinger et al., 1997 506).

Subsequently, mRNA was purified from 240μg of total RNA prepared as described in Section 2.8. The resulting mRNA purification procedure yielded 42.1μg mRNA at a concentration of 0.134μg/μl which constitutes 2.7% of the total RNA. The $A_{260/280}$ ratio of 2.03 again suggested that the mRNA sample was not contaminated with substantial amounts of protein, phenol or DNA.

This mRNA was then used to produce a cDNA library for the yeast 2-hybrid screen.

The presence of mRNA and also the presence of the 18S and 28S rRNA bands in total cellular RNA was verified using native agarose gel electrophoresis (Figure 3.1). The quality (degree of degradation) of RNA was evaluated by comparison of the intensities of the 18S and 28S rRNA bands. Since the 28S molecule is nearly 2.4 times longer (4.5kb *vs* 1.9kb) than 18S rRNA (Sobkiewicz and Twardowski, 1998), it is approximately 2.4 times more likely that randomly distributed endonuclease cleavage events would occur within it and would result in decreased intensity of the 28S band compared with 18S rRNA on the agarose gel. Therefore it is generally accepted that the ratio lower than 2:1 of 28S to 18S rRNA indicates degradation (Wang, 2005). There is a significant difference of intensity between the 28S and 18S rRNA bands (at least 2:1) which can be seen on the native agarose gel (lanes 3 and 4), suggesting that RNA was not degraded. mRNA appears as a smear on the gel. Note that traces of 28S and 18S rRNA seem to be present in the mRNA sample. However, the intensity of the rRNA appears to be several times lower in the purified mRNA than in the total RNA suggesting high enrichment of mRNA.

The degree of RNA degradation was further examined using denaturing agarose gel electrophoresis. An advantage of this approach is that denaturation disrupts the secondary and tertiary structures formed by rRNA (Streit et al., 2009). Since RNA forms complex three-dimensional structures which stabilise the folded molecule,

electrophoresis under native conditions may not reveal degradation of RNA, because even degraded RNA can still retain intact 3D structures due to the presence of weak bonds. Denaturation leads to linearization of RNA molecules and if degradation is present, it can be evaluated by comparison of the intensities of the 18S and 28S rRNA bands. Denaturing agarose gel electrophoresis (Figure 3.2) further confirms that the RNA is not degraded, as the ratio of 28S to 18S rRNA (lane 3, 5μg of total RNA loaded) was estimated to be at least 2:1. The mRNA smear is hardly visible on this denaturing agarose gel. However, under denaturing conditions, formation of dsRNA and, as a result, intercalation of ethidium bromide into the double stranded nucleic acids (thus, also fluorescence) is greatly reduced. Therefore, bands and smears composed of less abundant molecules, unlike bands composed of 18S and 28S rRNA's (which are very abundant in the cell), may not be readily visualized. Therefore the absence of a visible smear does not mean that mRNA is degraded or absent in the sample, it rather suggests that sensitivity of this technique or UV source used for visualization of RNA, is not sensitive or powerful enough to allow visualization of mRNA.

cDNA synthesis and library construction

cDNA synthesis is the process of generating DNA from an RNA template. Reverse transcriptase, an enzyme of retroviral origin that possesses an RNA directed DNA polymerase activity is used in this reaction. For the purpose of generating cDNA for the recombination steps involved in library construction, mRNA extracted from 786-0 cells was used for the first strand synthesis and was subsequently amplified by LD-PCR (long distance PCR, performed according to the Matchmaker Library Construction and Screening Kit manual) using primers specifically designed to allow

homologous recombination with the yeast vector pGADT7-Rec. The image shown in Figure 3.3 confirms the presence of amplified cDNA in the sample and the smear visible on the gel represents molecules of different sizes with an apparent average length of approximately 0.5-1kb. In the process of transformation, amplified cDNA and the linearized pGADT7-Rec vector are taken up by the competent yeast cells and recombination is carried out inside the yeast cells by cellular recombinases (Birnboim and Doly, 1979). Subsequent plating of the cells on –Leu DO agar Petri dishes allows selection for clones containing the circularized (circularization usually occurs by homologous recombination with the library cDNA) vector as shown in the Figure 3.4.

To evaluate the transformation efficiency (to ensure that the transformation efficiency was at least 1 x 10^6 per 3μg of the vector in order to provide a diverse and representative source of cDNA for the subsequent yeast 2-hybrid screen), a series of dilutions of the transformation reaction was performed. The recommended transformation efficiency had been experimentally determined by the manufacturer of the kit and the number of 10^6 appears to provide sufficiently high diversity of cDNA species in the library. Table 3.1 presents data regarding the actual transformation efficiency obtained, calculated from three different dilutions of the transformation reaction. The transformation efficiency thus determined was 1-2 x 10^6 clones per 1μg of pGADT7-Rec vector (in total 3μg of the vector was used for the library construction) based on the number of clones obtained on the –Leu DO agar medium. The lowest dilution, and therefore the highest number of colonies, is the least biased, therefore it is likely that the number of clones in the library per 1μg of plasmid DNA was around 1.8 x 10^6. This constituted an expected number of at least 10^6 independent clones in the library.

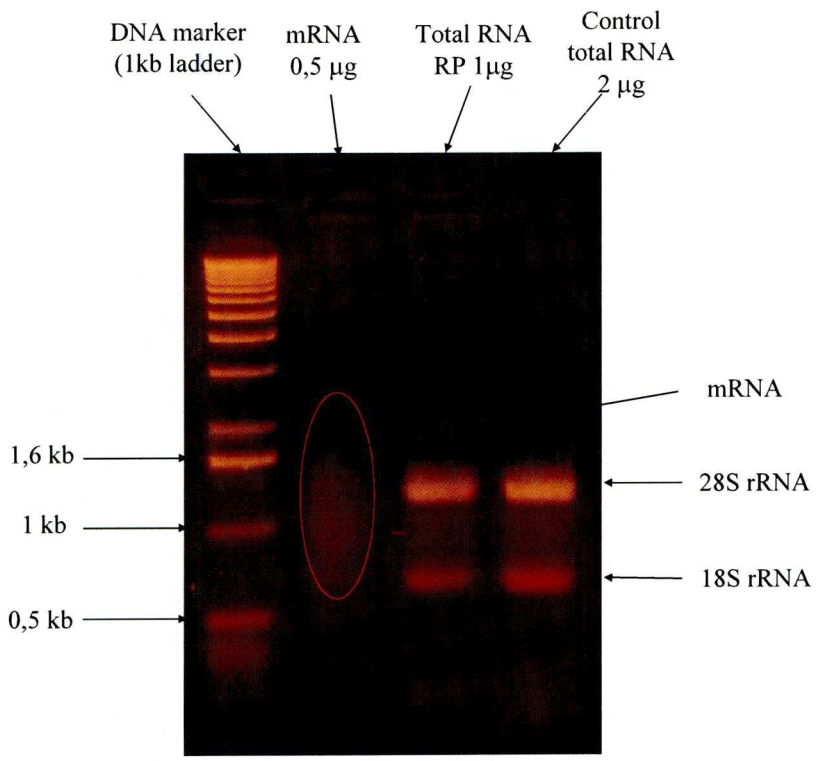


Figure 3.1 **Native 1.2% agarose gel electrophoresis of RNA.** mRNA appears as a smear in lane 2, next to the marker. Two strong bands corresponding to the 18S and 28S rRNA can be seen in lanes 3 and 4 where the total RNA samples were loaded. Note that traces of 28S and 18S rRNA seem to be present in the mRNA sample.

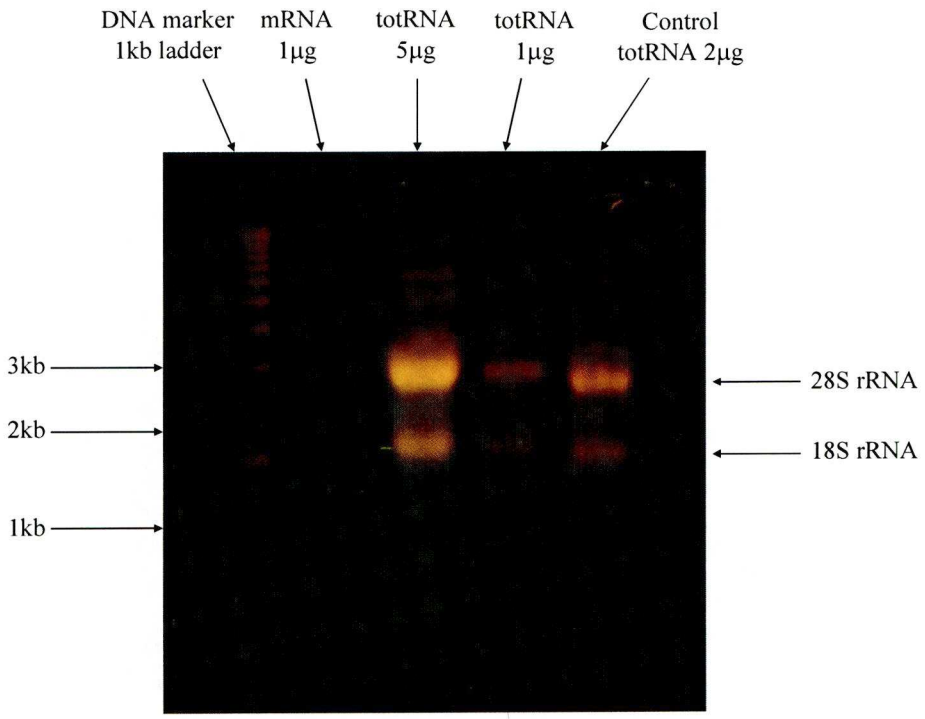


Figure 3.2 **Denaturing 1.2% agarose gel electrophoresis of mRNA and total RNA.** 1µg of mRNA, 5µg of total RNA, 1µg of total RNA and 2µg of a control total RNA were separated on the denaturing gel. Bands corresponding to the 18S and 28S rRNA are indicated.

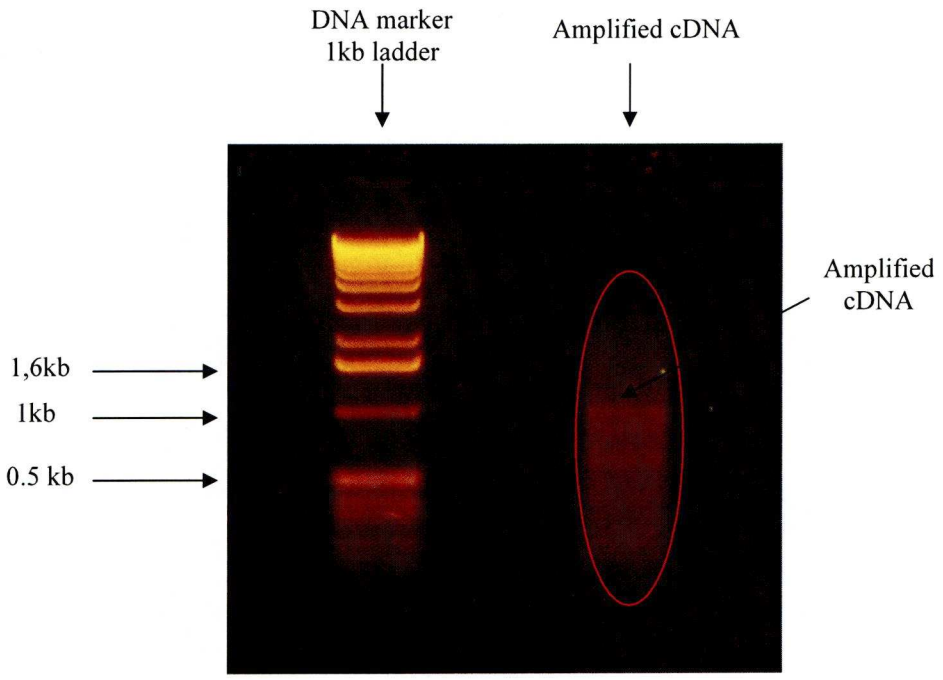


Figure 3.3 **Native agarose gel electrophoresis of amplified cDNA for library construction.** 7µl of the LD-PCR (long distance PCR) reaction (performed according to the Matchmaker Library Construction and Screening Kit manual) was loaded onto a 1.2% native agarose gel to visualise the products from the PCR reaction. 1µg of the DNA ladder marker was loaded on the gel; the 1.6kb band of the marker contains 100ng of DNA.

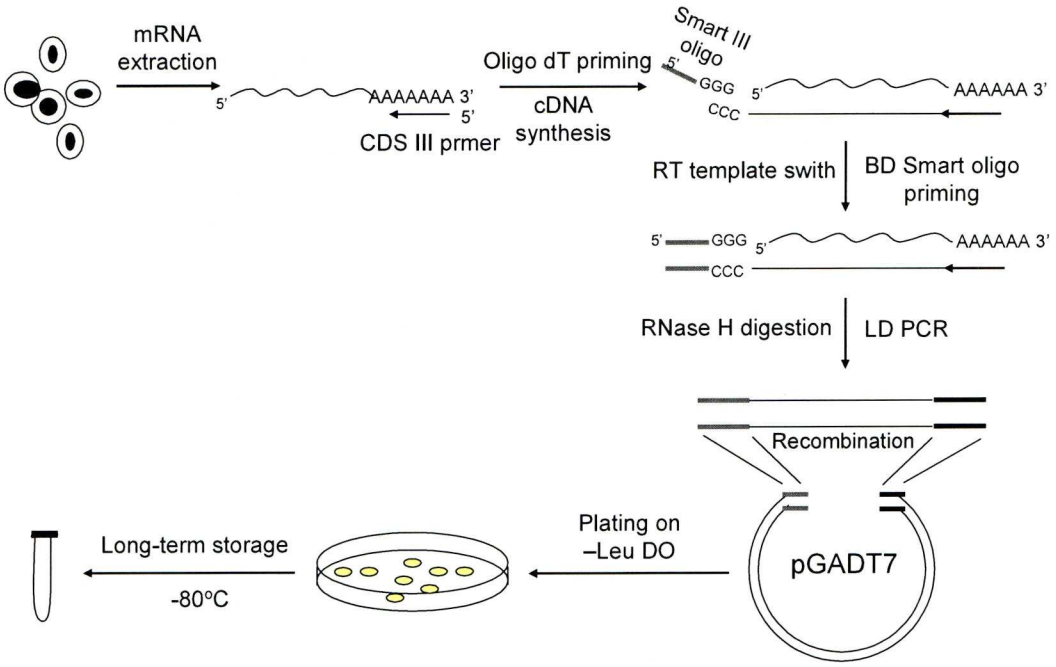


Figure 3.4 **Construction of the library.** The Figure presents several steps leading to generation of the library: RNA extraction from cells is followed by the first strand synthesis, long distance PCR which yields amplified cDNA for recombination with the library vector during the process of transformation into the yeast cells. The yeast clones which carry the library plasmids are selected on the –Leu DO plates, harvested, pooled and stored at -80°C.

Dilution of the library suspension	**No. of clones**	**Transformation efficiency (No. of clones/µg of pGADT7-Rec)**
1:100	180	1,.8 x 10^6
1:1,000	12	1.2 x 10^6
1:10,000	1	1.0 x 10^6

Table 3.1 **Library transformation efficiency.** The Table summarizes a series of dilutions performed in order to estimate the efficiency of transformation. The library suspension (total volume of 30ml) was diluted as shown in the first column and plated on the –Leu DO media. The third column shows transformation efficiency presented as a number of clones per one µg of plasmid DNA (out of 3µg of the total amount of the pGADT7-Rec vector used here) calculated based on the number of colonies (second columns) that grew on –Leu DO agar plates inoculated with the transformation mixture diluted as indicated in the first column.

3.1.2.2 Identification of putative MDM2 interacting proteins expressed in RCC cells

cDNAs from 722 clones obtained in the yeast two-hybrid screen were amplified by PCR, 178 of them were sequenced and identified using BLASTn (NCBI website). Out of 30 different sequences identified in the screen, 14 appeared to be present in a correct reading frame, 11 were out of frame and 5 corresponded to non-coding sequences. The screen reconfirmed previously described interactions of MDM2 with: ribosomal proteins L5, S7, L11, L26 and a transcription factor E2F1 which suggests that the technique reliably identified MDM2 interacting proteins. Several novel putative MDM2 interacting proteins were also identified.

Protein name	**Number of clones identified**	**Number of clones in a correct reading frame/number of clones tested**
Activating signal cointegrator 1 complex subunit 2	1	Non-coding sequence
Adhesion regulating molecule 1, transcript variant 2	1	0/1
Cyclophylin G	2	0/1
Cytochrome c, somatic	2	1/1
DnaJ(Hsp 40) homolog	1	0/1
E2F1	2	2/2
General transcription factor IIF	1	1/1
H2A histone family, member Z	1	0/1
Hipothetical protein MGCG1	3	3/3
L1	1	1/1
L3	4	0/2
L11	2	2/2
L12	1	1/1
L26	11	5/5
L27	2	0/2
L31	9	3/3
L36, L36-like, similar to L36a	72	10/10
Lectin, mannose-binding-2	1	0/1
Mitochondrial genomic sequence	4	Non-coding sequence
NME2	2	0/2

Peptidase alpha (mitochondrial processing)	1	Non-coding sequence
Pseudouridilate synthase-like 1	6	0/1
S7	37	7/7
S23	2	2/2
S25	3	3/3
S27a	2	1/1
Small ribonucleoprotein D2 polypeptide transcript variant 1	1	Non-coding sequence
Thioredoxin reductase 2	1	0/1
Ubiquinol-cytochrome c reductase complex, transcript variant 1	3	0/1
Y box protein	1	Non-coding sequence

Table 3.2 **Putative MDM2 interacting proteins identified in the yeast two-hybrid screen.** The Table summarizes the screen results with clones listed in alphabetical order. The middle column shows the number of clones obtained in the screen and the right-hand column shows the number of clones that proved to be in a correct reading frame with the activation domain of the Gal4 transcription factor expressed as a fraction of the total number of clones checked for in-frame compatibility with the pGADT7-Rec vector.

3.1.3 Re-testing of the NME2 interaction with MDM2 and the β-galactosidase assay confirm screen results

Two clones encoding the NME2 protein were identified in the yeast two-hybrid screen. NME2 was chosen for further analysis because it was independently identified as an MDM2 interacting protein in our laboratory using a proteomic approach - NME2 present in the HEK293 cell lysate, appeared to bind to His-tagged MDM2 coating the nickel-sepharose column (Dr Maria Maguire, unpublished results). However, at the time when this decision was taken, it was not apparent that both NME2 clones identified in the screen were not recombined with the pGADT7-Rec vector in a correct reading frame, therefore potentially producing a false positive result. However, published data suggest that an out of frame construct may produce a protein of a correct amino acid sequence. This issue will be discussed more extensively in Section 4.2. To authenticate the screen result, the MDM2 – NME2 interaction was retested in yeast with full length NME2. cDNA of an orthologous, highly related gene (encoding a protein exhibiting 88% amino acid identity), *NME1*, was also cloned into the pGADT7 vector and tested.

The yeast two-hybrid assay measures the ability of two proteins to interact with each other in the yeast system. A positive result is manifested by yeast growth and activation of reporter genes; MEL1 (encoding α-galactosidase degrading the X-α–gal to a blue product which is detected on the X-α-gal agar plates) and β-galactosidase (used for quantitative analysis of the strength of the interaction in the β-galactosidase assay). As Figure 3.5 shows, NME2 interacts with MDM2 in this system. Although yeast clones expressing the NME2 and MDM2 fusions grew much slower than the p53 and Large T expressing yeast (used as a positive control for protein-protein interaction), the colonies and adjacent agar medium (supplemented

with X-α-gal) appeared blue, suggesting that a sufficiently strong interaction occurred between NME2 and MDM2 to activate the MEL1 reporter gene. Figure 3.5 also shows, that NME1 did not detectably interact with MDM2 as no growth could be observed in yeast expressing these proteins. These results suggest that NME2 interacts with MDM2 in yeast, and that this interaction is highly specific, since the highly homologous NME1 protein (see Figure 4.2) did not give a positive signal in the yeast two-hybrid assay.

The β-galactosidase assay, which measures the activity of the second reporter gene, β-galactosidase (due to its ability to degrade X–β–gal to a product which can be colorimetrically assayed) revealed that the NME2-MDM2 interaction leads to induction of the β-galactosidase activity by nearly three-fold compared to the yeast lysate control. However, it does not appear to be as active as the positive control (Large T - p53) which induced the activity of the β-galactosidase by ten-fold compared to the yeast lysate control. Thus, the β-galactosidase assay further supports the results obtained on the plate and shows activation of an additional, to MEL1, reporter gene as a result of the NME2-MDM2 interaction, as well as yielding quantitative results.

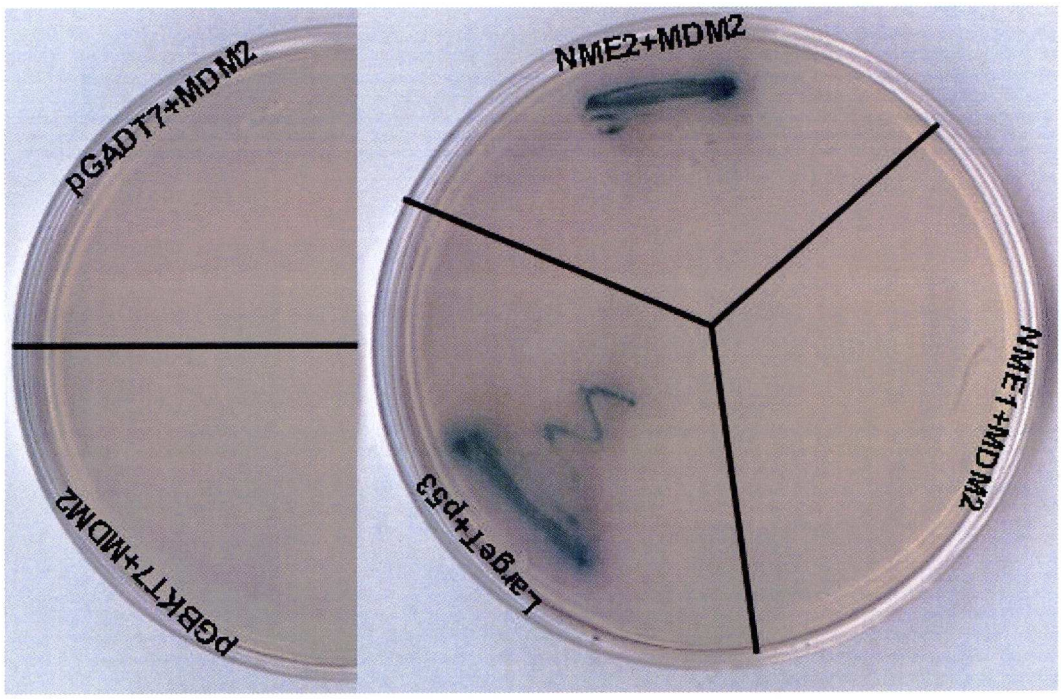


Figure 3.5 **The yeast two-hybrid assay for interactions of MDM2 with NME1 and NME2 shows that NME2, but not NME1 interacts with MDM2.** The Figure shows yeast cells plated on QDO agar media supplemented with X-α-gal. The interaction between NME2/NME1 and MDM2 was tested in this experiment. Growth and blue colour indicate that there is an interaction between NME2 and MDM2 and the positive control of p53 and LargeT. In contrast, neither the control nor the NME1-MDM2 pairs appeared to promote yeast growth and activate the reporter gene in this assay.

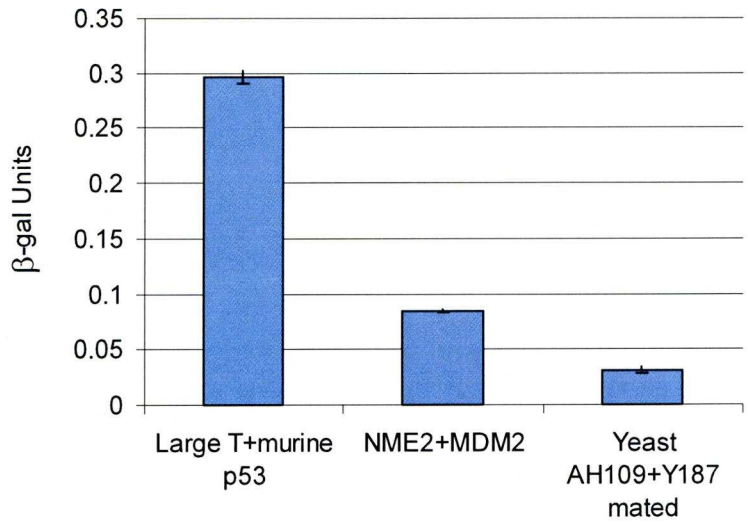


Figure 3.6 **β-galactosidase assay of the NME2-MDM2 interaction.** β-galactosidase assay demonstrates that NME2 interacts with MDM2 in yeast. The Figure shows β-galactosidase activity in cell lysates obtained following a yeast two-hybrid assay for NME2 interaction with MDM2 and for p53 interaction with Large T antigen for comparison as a positive control. The lysate from mated AH109 and Y187 yeast was used as a negative control. 1 unit of β-galactosidase catalyses hydrolysis of 1μmol of ONPG (ortho-Nitrophenyl-β-galactoside) per minute at pH=7.5 at 37°C.

3.1.4 Functional assays reveal that NME2 regulates the activity of p53

The yeast two-hybrid system is a sensitive technique that allows high throughput screening. It also serves as a useful tool for testing and analysis of protein-protein interactions, yielding quantitative results when the β-galactosidase assay is performed.

There are, however, several drawbacks of this system. Cytoplasmic and trans-membrane proteins may not adopt a proper conformation in the nuclear environment. Several post-translational modifications determining conformation and functionality of proteins in higher eukaryotes may be different in yeast. Hence, false positive and negative results do occur. Therefore, yeast two-hybrid results should be confirmed using other methods such as in vitro binding assays, coIP or a mammalian two-hybrid assay to increase confidence that the interaction detected in yeast is real. A good way of verifying the interaction may be a functional assay which depends on protein-protein interactions. One way to perform a functional assay is co-over-expression of two or more proteins in cells and subsequent observation if their levels or functions change in comparison with control samples.

The most sensitive assay reflecting changes in the activity of MDM2 available in our laboratory, is a luciferase assay which measures the activity of p53. This assay, along with protein analysis by western blotting, was used in the following series of transfection experiments in order to determine functional consequences of the NME2-MDM2 interaction.

NME2, together with p53 and MDM2 were transfected into three different cell lines: H1299 (non small cell lung cancer, p53-null cell line), MCF-7 (breast cancer cell line, p53 wt) and BJ-fibroblasts (human fibroblasts). These cells were chosen for transfection because they are either p53-null (H1299) and therefore suitable for

experiments using exogenous p53; or expressing wt p53 (MCF-7 and BJ-fibroblasts) allowing evaluation of the effects of transfected NME2 on the levels/activity of the endogenous protein. Additionally, this selection allowed use of already optimised transfection protocols, as these cell lines are commonly used in our laboratory. The added benefit of using BJ fibroblasts was the possibility of testing of the functional consequences of the NME2-MDM2 interaction in non-cancer cells. To evaluate the influence of NME2 on the activity of p53 and MDM2, NME2 was co-transfected with p53 and MDM2 (according to the protocol described in Section 2.17). The activity of p53 was measured using the luciferase assay and protein levels analysed by western blotting.

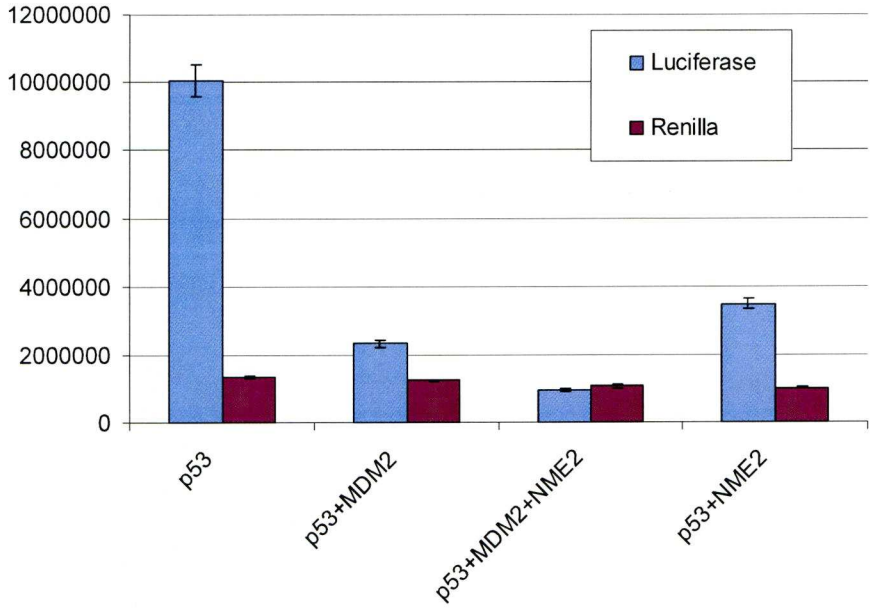
The experiments show that transfection of NME2 leads to a decrease in p53 transcriptional activity in the absence of exogenously expressed MDM2 and augments the decrease of p53 activity in the presence of exogenously expressed MDM2 in all three tested cell lines (Figures 3.7, 3.8, 3.9). The activity of the endogenously expressed p53 does not seem to be affected in MCF-7 cells (Figure 3.8), however, NME2 expression appears to reduce the activity of endogenous p53 in BJ fibroblasts (Figure 3.9).

Western blot analysis shows that, although the NME2-dependent reduction of the activity of p53 is not accompanied by reduction in p53 levels, NME2 seems to have an effect on the MDM2-dependent post-translational modification of p53 (intensity of the ladder of more slowly migrating forms of p53 decreases upon co-transfection with NME2) in H1299 (Figure 3.7) and MCF-7 (Figure 3.8). The blot for MDM2 indicates that NME2 may have an effect on the steady state level of MDM2 which

seems to be lower in the sample co-transfected with NME2 than for the sample without NME2.

The results therefore suggest that NME2 may have pleiotropic effects on the p53-MDM2 network as it reduces the activity of p53 in both presence and absence of exogenously expressed MDM2, changes the MDM2-dependent post-translational modifications of p53 and, potentially, also reduces the level of MDM2.

a)



b)

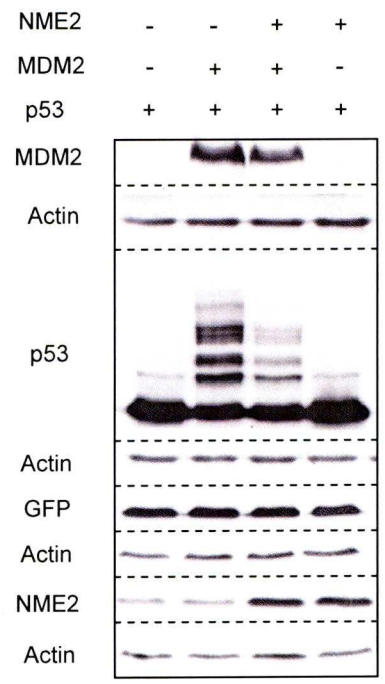
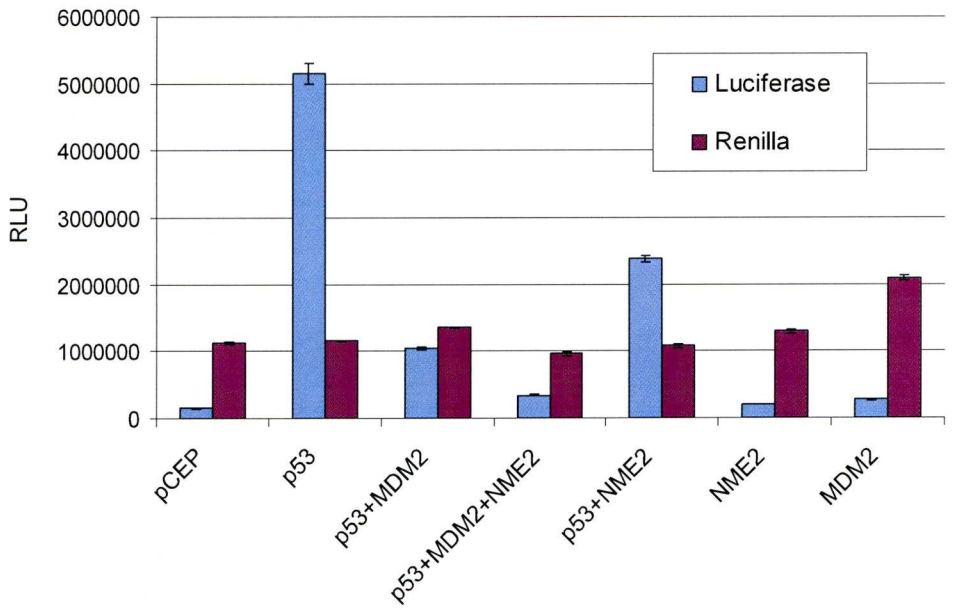


Figure 3.7 **NME2 decreases the activity of p53 and the intensity of the post-translational modification of p53.** The H1299 cells were seeded into six-well plates and after 24h transfected with 0.02μg of pCEP-p53, 0.06μg of pCMV-neo-bam-

MDM2, 3μg of pCEP-NME2 and 0.3μg of pSUPER-GFP per one well of the six-well plate in triplicates, using Genejuice as a transfection reagent at a ratio of Genejuice to DNA 3:1 (the procedure described in Section 2.18). Note that small amounts of the p53 and MDM2 plasmid DNA were used compared with the amount of NME2 in order to avoid apoptosis in transfected cells and to guarantee sensitivity of the system (the effect of NME2 on the activity of p53 could be less obvious if large amounts of p53 were used; additionally, larger amounts of MDM2 would lead to significantly more decreased activity of p53 so that further suppression due to the action of NME2 would not be detectable). 24h after transfection, cells from three wells (of the 6-well plate) were harvested for protein analysis by western blotting and cells from the remaining three wells were lysed for the luciferase assay. a) Histogram shows the results of the dual luciferase reporter assays of the activity of p53 performed in the H1299 cells. The activity of luciferase or renilla was measured in the cell lysates (method described in Section 2.17) as indicated, normalised to protein concentration and the mean of three replicates +/- s.e.m is presented on the histogram as RLU (relative light units). b) Western blot analysis of transfected samples show main bands of p53 and its modified forms, MDM2 and NME2. Actin was used as a loading control and GFP indicates transfection efficiency.

a)



b)

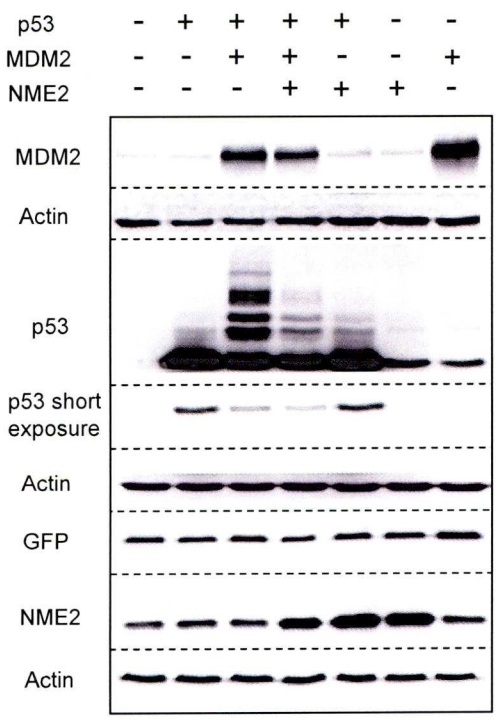


Figure 3.8 **NME2 decreases p53 activity in the MCF-7 cell line.** The MCF-7 cells were seeded into six-well plates and after 24h transfected, harvested and assayed by western blotting and subject the luciferase assay as described previously (see legend for the Figure 3.7)

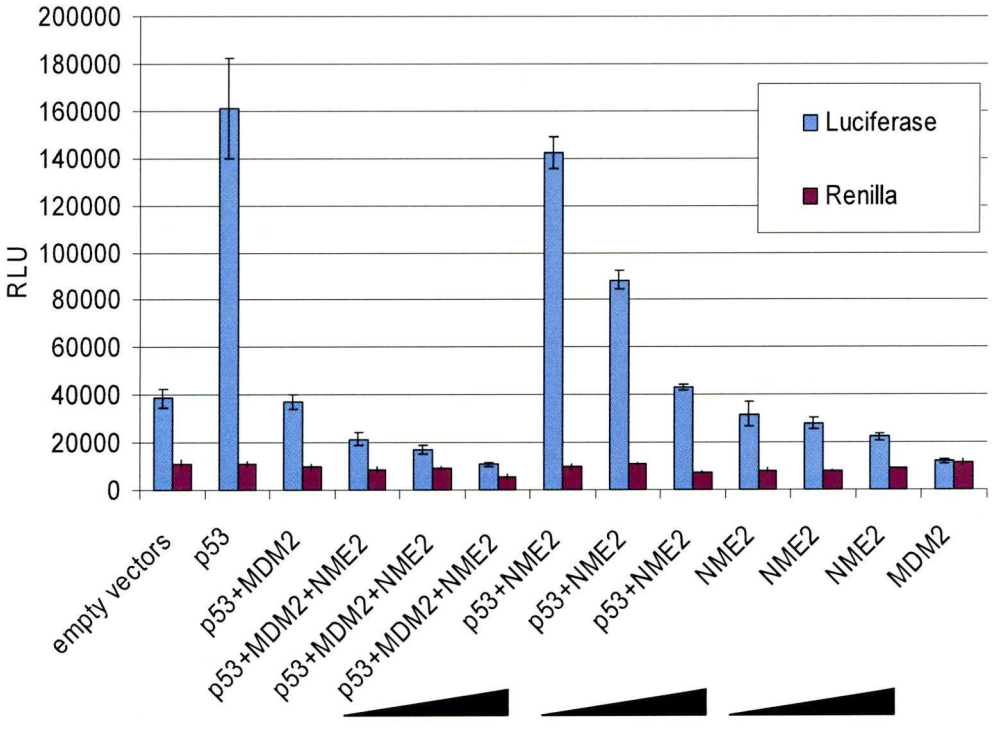


Figure 3.9 **Titration of NME2 in BJ fibroblasts shows the dose-dependent decrease of p53 activity.** In this experiment, NME2 was titrated in the presence and absence of exogenously expressed MDM2 and the activity of both transfected and endogenous p53 was measured. BJ-fibroblasts were seeded into six-well plates and after 24h transfected with 0.04μg of pCEP-p53, 0.04μg of pCMV-neo-bam-MDM2 and 0.3μg, 1μg and 3μg of pCEP-NME2 per one well of the six-well plate in triplicates, using Genejuice as a transfection reagent at a ratio of Genejuice to DNA 2.5:1 (according to the procedure described in Section 2.18). All subsequent steps were carried out essentially as described in the legend for the Figure 3.7.

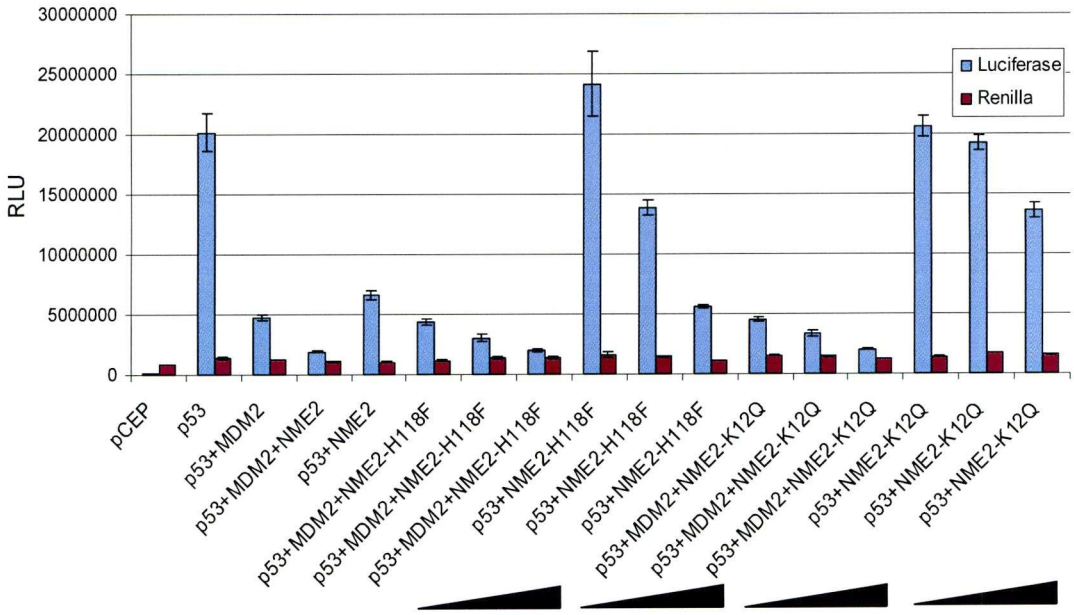
Note that no western blot control was performed, as the transfection efficiency of BJ fibroblasts is no higher than 1% as determined by the *in situ* β-galactosidase assay and thus the endogenous levels of the proteins mask the signal coming from the transfected protein.

3.1.5 The ability of NME2 to reduce the activity of p53 is not dependent on its kinase activity

NME proteins predominantly function as nucleoside diphosphate kinases (reviewed in Lacombe et al., 2000) and this function could be vital for NME2 to be able to reduce the activity of p53. To investigate whether reduction of the activity of p53 upon NME2 transfection is dependent on the kinase activity of NME2, a H118F kinase mutant (described in Postel et al., 1996) was constructed and tested. Additionally, another mutant, K12Q (K12Q mutation abolishes all known functions of NME2 (Postel et al., 2002), was also constructed and tested in these experiments.

Wt protein and the kinase mutant (H118F) of NME2 decrease the activity of p53 in the presence and absence of exogenously expressed MDM2 as shown in Figure 3.10a. The ability of the "total loss of function" NME2 mutant (K12Q) to reduce the activity of transfected p53 appears to be only slightly compromised suggesting that none of the other previously identified activities of NME2 determine this effect and this is compatible with a requirement for direct protein-protein interaction to mediate the effect of NME2 on p53. The western blot (Figure 3.10b) shows that the transfection efficiency between the samples and the expression level of MDM2, p53 and NME2 proteins are essentially even. The result therefore suggests that the kinase activity of NME2 is dispensable for the ability of NME2 to inhibit p53.

a)



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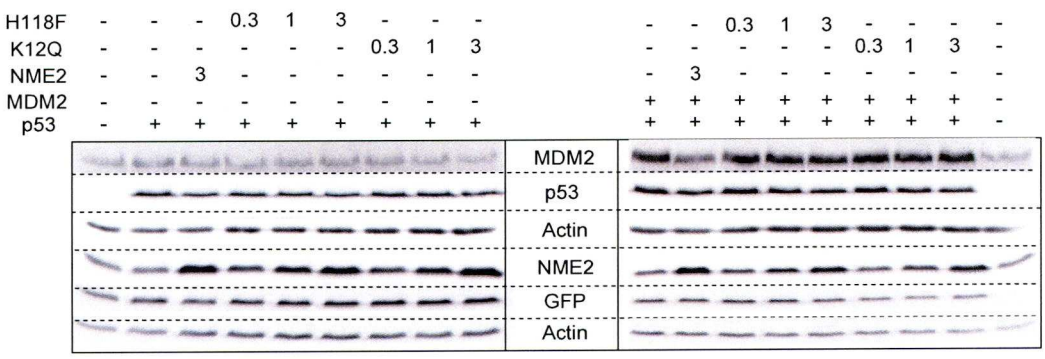


Figure 3.10 **The effect of mutant-NME2 proteins on the activity of p53.** The experiment was performed and the data are presented essentially as described in the legend for the Figure 3.7 and presents the luciferase assay (a) and western blot analysis (b).

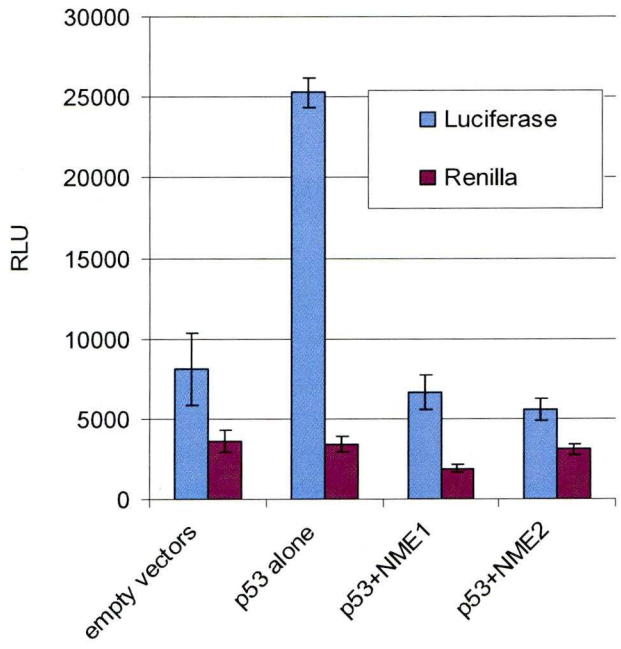
3.1.6 NME1 and NME2 inhibit the activity of p53 but differentially regulate the levels of MDM2

NME2 shares 88% homology at the protein level with its ortholog, NME1 (Figure 4.1). Since NME2 but not NME1 interacted with MDM2 in yeast, the ability of NME1 to negatively regulate the activity of p53 was examined in BJ fibroblasts (Figure 3.11a) and in p53/MDM2-double null ($Mdm2^{-/-}$, $p53^{-/-}$) MEFs (Figure 3.11b) using the luciferase assay. BJ fibroblasts (3.11a) proved to be a reliable non-cancer cell line for studying the effects of NME2 on the activity of p53 (Figure 3.9) and therefore these have also been used to study the effect of NME1 on the activity of p53. Additionally, as it was shown that NME2 reduced the activity of p53 in absence of exogenously expressed MDM2 (Section 3.1.4), it was not clear if this effect was mediated through the action of the endogenously expressed MDM2, or MDM2-independently. There are detectable levels of endogenously expressed MDM2 present in H1299, MCF-7 and BJ-fibroblast cells and it is possible, that NME2 could act through them to decrease the activity of p53. Thus a major advantage of using the double null MEFs was the possibility to determine whether the NME2 (and possibly NME1) mediated suppression of the activity of p53 depends on MDM2 protein.

The results of the experiment shown in Figure 3.11 demonstrate that NME1 co-transfected with p53 leads to a reduction of the activity of p53 to a similar extent to that observed for NME2 (Figure 3.11a). Moreover, (as Figure 3.11b illustrates) the experiment performed in double null MEFs shows that in absence of MDM2 both NME1 and NME2 are capable of reducing the activity of p53. The results therefore suggest that p53 inhibition is an MDM2-independent property of NME1 and NME2 and that the protein sequence differences between NME1 and NME2 do not affect their ability to suppress p53 transcriptional activity.

Previous experiments (see Section 3.1.4, Figures 3.7 and 3.8) suggested a possible effect of NME2 on the levels of the MDM2 protein. To investigate this issue, NME1, NME2, and the K12Q mutant of NME2 were co-transfected with MDM2 into H1299 cells. NME2 appears to down-regulate expression of MDM2 and the decrease of MDM2 levels does not occur when NME1 is co-expressed with MDM2 (Figure 3.12). The K12Q mutant appears to promote an intermediate level of MDM2 protein and thus it appears that this mutant of NME decreases the level of MDM2 less efficiently than the wt protein. The results suggest that unlike NME1, NME2 could be involved in regulating MDM2 steady state levels.

a)



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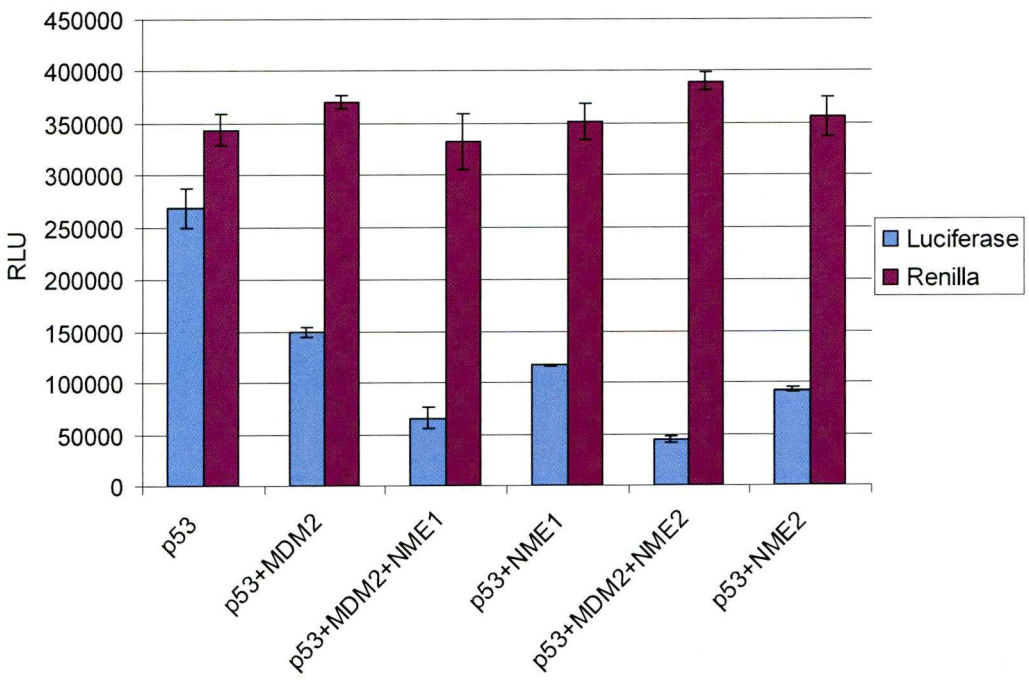


Figure 3.11 **Comparison of the ability of NME1 and NME2 to decrease the activity of p53 in normal cells and in the MDM2-null background.** In this experiment, the ability of NME1 and NME2 to reduce the activity of p53 in BJ

fibroblasts (a) and p53/MDM2-double null ($Mdm2^{-/-}$, $p53^{-/-}$) MEFs (b) was compared. The BJ fibroblasts and MEFs cells were seeded into six-well plates and after 24h transfected with 0.02μg of pCEP-p53, 0.06μg of pCMV-neo-bam-MDM2 (b only) and 3μg of pCEP-NME2 per one well of the six-well plate in triplicates as indicated, using Genejuice as a transfection reagent at a ratio of Genejuice to DNA 2.5 : 1 (the procedure described in Section 2.18). All subsequent steps were carried out essentially as described in the legend for the Figure 3.7. Note that no western blot control was performed, as the transfection efficiency of fibroblasts is no higher than 1% as determined by the *in situ* β-galactosidase assay and thus the endogenous levels of the proteins mask the signal generated by the transfected protein.

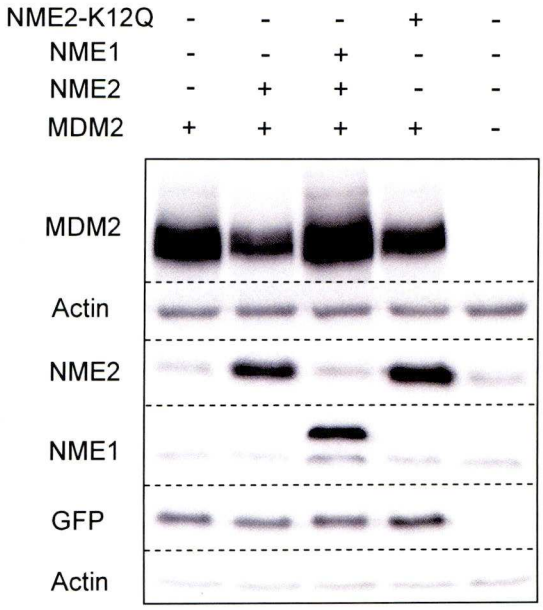


Figure 3.12 **NME2 downregulates MDM2.** The Figure shows western blot analysis of H1299 cells transfected with MDM2 and NMEs. The H1299 cells were seeded into six-well plates and after 24h transfected with 0.5μg of pCMV-neo-bam-MDM2, 3μg of pCEP-NME1/2/K12Q and 0.3μg of pSUPER-GFP per one well of the six-well plate in triplicates essentially as described in the legend for the Figure 3.7 except, that only western blotting was performed. Note that large amounts of the MDM2 plasmid DNA were used compared with these used previously (see Sections 3.1.4, 3.1.5 and 3.1.6) as it was experimentally defined that higher levels of MDM2 enable better visualization of the NME2-mediated effect than low levels of MDM2.

3.1.7 The reduction of MDM2 upon co-expression with NME2 is dependent on the activity of 26S proteasomes, an intact RING-finger of MDM2 and the N-terminus of NME2

It was previously shown (Figure 3.12) that transfection of NME2 led to a decrease in the MDM2 protein levels, however, it remained unclear if this was due to changes in transcription, translation or stability of MDM2. To determine whether the observed reduction in MDM2 levels was mediated *via* the proteasomal degradation pathway, the proteasome inhibitor MG132 was used to inhibit degradation.

As Figure 3.13 shows, NME2-dependent down-regulation of MDM2 appears to be inhibited by the proteasome inhibitor. Additionally, the K12Q mutation does not seem to abrogate the ability of NME2 to down-regulate MDM2 suggesting that this is a novel activity of NME2 (as the K12Q mutation has been shown to abrogate all known functions of NME2 (Postel et al., 2002). Since reduction of the MDM2 level upon co-transfection with NME2 appears to occur *via* the proteasomal pathway, it could therefore be hypothesized that this is a result of increased auto-ubiquitylation of MDM2 (MDM2 is known to possess an E3 ubiquitin ligase activity that can promote auto-ubiquitylation and degradation as described in Section 1.5.4.1.3).

Therefore, a RING-finger mutant of MDM2 (which is unable to auto-ubiquitylate), or wt MDM2 were co-transfected with NME2 to test if the NME2-dependent decrease in MDM2 levels is dependent on the activity of the RING domain of MDM2. The results show (Figure 3.14) that the RING-finger mutant of MDM2, unlike the wt protein, is refractory to both NME2- and NME2-K12Q - induced down-regulation. NME1 does not appear to influence the level of MDM2 suggesting that reduction of MDM2 levels upon co-expression of NME2 might be a result of protein-protein interaction dependent on an intact RING-finger domain of MDM2.

Additionally, as shown on the p53 blot (Figure 3.14), p53 appears to undergo post-translational modifications which are dependent on the intact RING-finger domain of MDM2 as the RING finger mutant (MDM2-C464A) does not appear to promote, nor undergo such modifications. The migration pattern of the modified p53 detected on the western blot suggests poly-modification (attachment of chains of proteins, such as ubiquitin), or multiple mono-modification (by monomeric modifiers such as ubiquitin at several different lysine residues) as several different bands of gradually increasing molecular weight are visible. NME2 or its kinase mutant H118F decrease the intensity of the fraction of high molecular weight forms of modified p53 (most likely corresponding to the poly-modified p53) and seem to increase the intensity of the first few bands of the ladder (which the most likely correspond to p53 which is mono-modified at a few different residues). This suggests that NME2 could promote changes in the MDM2-mediated modification of p53 by altering its ability to promote its poly- or mono-modification. Moreover, the results suggest that the kinase activity of NME2 is dispensable for this process as the H118F mutant of NME2 also leads to changes in the pattern of p53 modification.

In order to establish which part of NME2 is responsible for the NME2-mediated reduction of the MDM2 level, chimeric NME1/2 proteins were generated (Section 2.14) and expressed in H1299 cells. As the majority of differences in the amino acid sequence between NME1 and NME2 are within the N-terminus, the N-terminus of NME2 was substituted with the N-terminus of NME1 (1+2). Accordingly, in the second chimeric protein, the C-teminus of NME2 was substituted with the C-terminus of NME1 (2+1). The ability of NME2 to decrease the level of MDM2 is conferred by its N-terminus (Figure 3.15). The chimeric protein 1+2, unlike the 2+1 chimera, does not have the ability to decrease the level of MDM2.

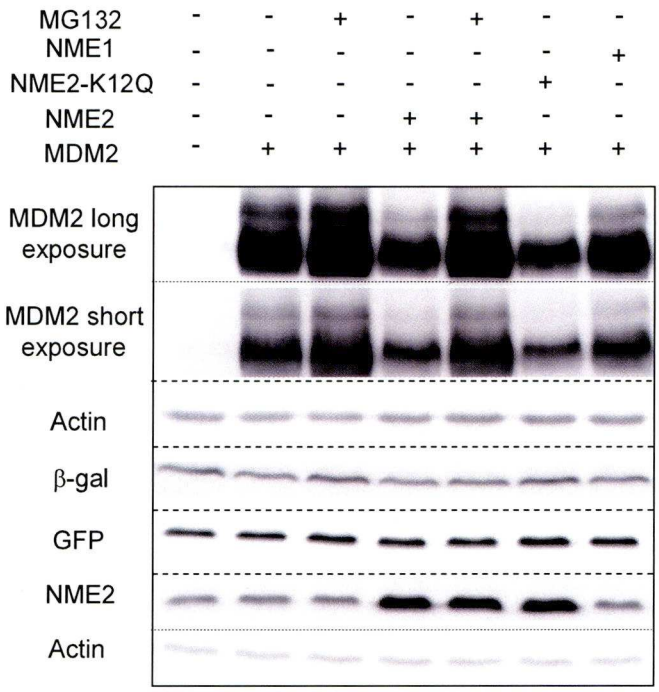


Figure 3.13 **Inhibition of the 26S proteasome rescues the NME2-mediated decrease of the levels of MDM2.** The Figure shows western blot analysis of H1299 cells transfected with MDM2, NME1, NME2 and NME2-K12Q as indicated. H1299 cells were seeded into six-well plates and after 24h transfected with 0.5μg of pCMV-neo-bam-MDM2, 3μg of pCEP-NME1/2/K12Q and 0.3μg of pSUPER-GFP per one well of the six-well plate essentially as described in the legend for the Figure 3.7. 19h after transfection, cells were treated with the proteasome inhibitor MG132 at 10μM concentration for 5h and cells were then harvested for western blotting. Note that large amounts of the MDM2 plasmid DNA were used for reasons explained in the legend for the Figure 3.12.

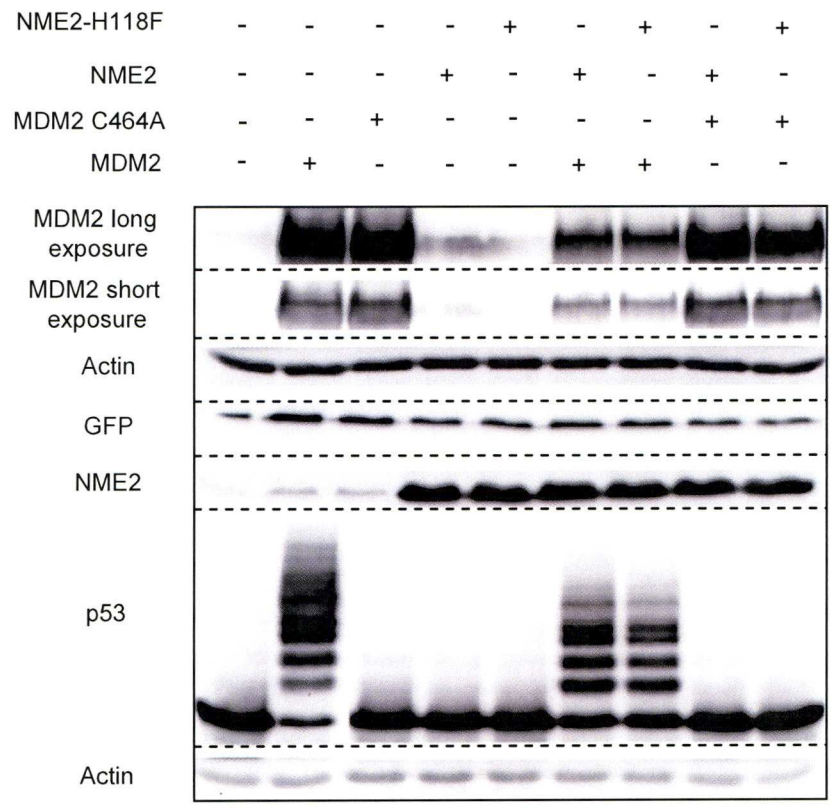


Figure 3.14 **NME2 has no effect on the RING-finger mutant of MDM2**. The Figure shows western blot analysis of H1299 cells transfected with p53, MDM2, MDM2-C464A (RING-finger mutant), NME2 and NME2-H118F. The H1299 cells were seeded into six-well plates and after 24h transfected with 0.083μg of pCEP-p53, 0.5μg of pCMV-neo-bam-MDM2/C464A, 3μg of pCEP-NME2/H118F and 0.3μg of pSUPER-GFP per one well of the six-well plate in triplicates, essentially as described in the legend for the Figure 3.7 except, that only western blotting was performed. Note that large amounts of the MDM2 plasmid DNA were used for reasons explained in the legend for the Figure 3.12.

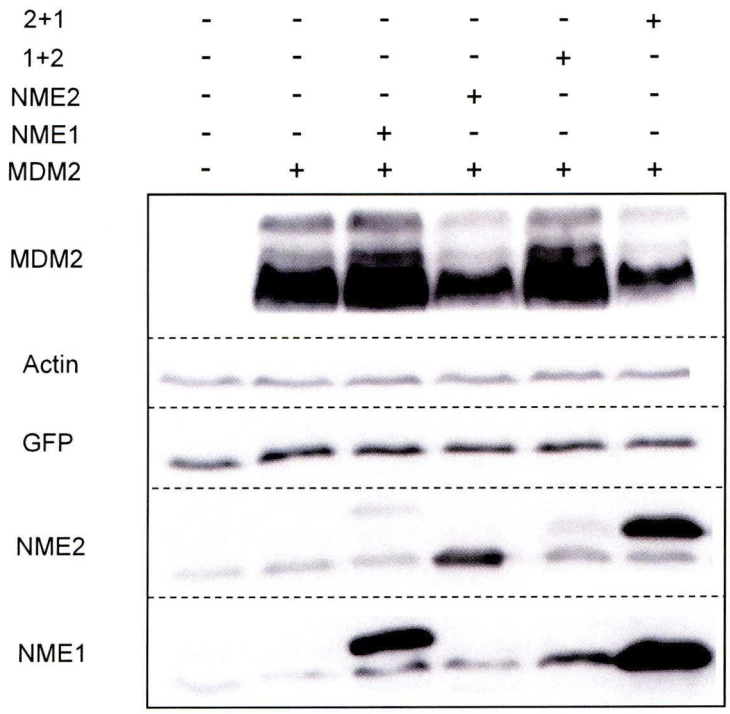


Figure 3.15 **N-terminus of NME2 is responsible for the reduction of the MDM2 expression levels**. The figure shows western blot analysis of H1299 cells transfected with MDM2, NME2 and NME2 chimeric expression constructs 1+2 (N' of NME1 fused to C' of NME2) and 2+1 (N' of NME2 fused to C' of NME1). The H1299 cells were seeded into six-well plates and after 24h transfected with 0.5μg of pCMV-neo-bam-MDM2, 3μg of pCEP-NME constructs and 0.3μg of pSUPER-GFP per one well of the six-well plate in triplicates, essentially as described in the legend for the Figure 3.7, except that only western blotting was performed. Note that large amounts of the MDM2 plasmid DNA were used for reasons explained in the legend for the Figure 3.12.

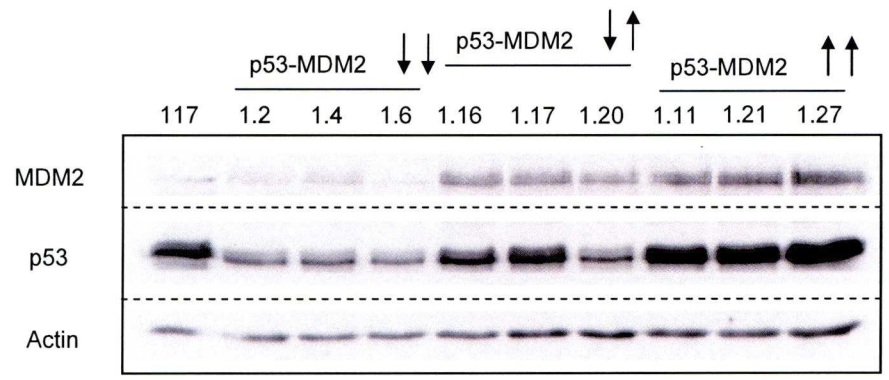
3.2 MDM2 and p53 increase the motility of RCC cells

The results presented in previous sections clearly suggest pleiotropic involvement of NME2 in the regulation of p53 and MDM2. This may indicate a role for NME2 in the fine-tuning of the p53-MDM2 network; however, such data do not explain the situation present in RCC, where over-expression of both p53 and MDM2 results in poor outcome as it has been shown that NME2 is rather down-regulated in high grade tumours (see Section 4.5). Here, no obvious effects of p53 and MDM2 could be seen on the levels of NME2. Therefore, the next step in this project aimed at identification of a process or mechanism that engages all three proteins: NME2, MDM2 and p53 and which might better account for the association between p53, MDM2 and outcome in RCC. NME2 has been described previously to function as a motility and metastasis suppressor (see Section 4.3). We therefore investigated whether p53 and MDM2 play a role in the regulation of motility in RCC cells and moreover, whether any such effect might act via NME2. Cellular motility plays a crucial role in processes such as development (migration of precursor cells) and in immunity. On the other hand, increased motility in cancer cells correlates with cancer progression, since the cells can more actively migrate and colonize distant tissues (Rinker-Schaeffer et al., 1996). An in vitro model of RCC tumour progression (a surrogate of the situation observed in RCC, where cells spontaneously acquire high levels of wt p53 and MDM2, see Section 2.21) was used in this study. The *in vitro* model of RCC tumour progression is based on derivative clones of the 117 cell line which naturally express low levels of p53 and MDM2, which we refer to hereafter as "double low": 1.1, 1.2, 1.6; clones expressing intermediate levels of p53 and MDM2, "double intermediate": 1.16, 1.17, 1.20; or clones spontaneously over-expressing both proteins, "double high" 1.11, 1.21, 1.27. These cell lines were

examined using Boyden chambers to determine their motility (an *in vitro* assay that reflects one aspect of the cells' metastatic potential) to evaluate and compare their motility.

The ability of "double low" and "double intermediate" cells to migrate through the pores of the membrane appears to be unchanged in comparison with the parental 117 cell line. "Double high" cells however, were highly motile in this experiment (Figure 3.16). This suggests a possible involvement of p53 and/or MDM2 in induction of motility in RCC cells.

a)



b)

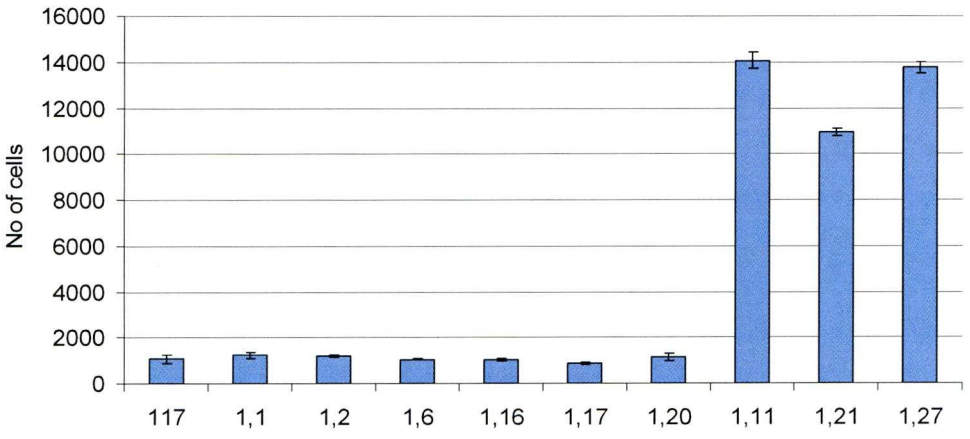


Figure 3.16 **Basal levels of protein expression and motility of 117 derivative cells.** The 117 parental cell line and derivative cell lines were harvested and subject to western blotting analysis and motility assay. The panel above (a) presents western blot analysis of p53 and MDM2 expression in 117 RCC cell lines. Actin was used as a loading control. b) The histogram shows analysis of the Boyden chamber motility assay performed essentially as described in Section 2.20. Cells were harvested and counted, and then 50,000 cells were seeded into each chamber. After 18h the cells were carefully wiped of the inner side of the porous membranes of Boyden chambers. Cells were subsequently fixed and stained. The membranes were then

mounted on the slide, covered with a cover slip and the cells were counted. The number of cells that migrated through membranes of Boyden chambers are presented on the histogram as the mean of three replicates +/- s.e.m. In this experiment, the parental 117 cell line was compared with three "double low" (1.1, 1.2, 1.6), three "double intermediate" (1.16, 1.17, 1.20) and three "double high" (1.11, 1.21, 1.27) 117-derivative cell lines (for details regarding 117-derivative cell lines see Section 2.21).

3.2.1 p53 increases motility by promoting over-expression of MDM2

As shown in the previous experiment, RCC cells over-expressing p53 and MDM2 were characterised by increased motility. We therefore set out to investigate whether this phenotypic change was a result of up-regulated p53 and/or MDM2. Firstly, transient reduction of p53 levels was achieved using siRNA in the p53 and MDM2 over-expressing 1.21 and 1.27 RCC cell lines and the Boyden chamber motility assay was again performed. In both cell lines, transfection of p53-siRNA results in decreased motility. The western blot shows that the p53 levels were successfully reduced upon siRNA treatment, as the intensity of p53 bands decreases in p53-siRNA treated samples compared to the scrambled siRNA control. Interestingly, intensity of the MDM2 bands in the samples treated with p53-siRNA is also reduced suggesting that high levels of MDM2 might be promoted by p53 in these cells (Figure 3.17). It was still not clear, however, whether increased motility in these "double high" cells was due to over-expression of p53 itself or rather was a result of p53-promoted MDM2 up-regulation. To address this issue, MDM2 and p53 were reduced using siRNA individually and simultaneously in five "double high" cell lines: 117 derivative, A498 and Caki-2. Cell motility was again measured using Boyden chambers.

These experiments show that depletion of either p53 or MDM2 results in decreased motility, but depletion of MDM2 seems to generally have a more pronounced effect (Figure 3.18). Interestingly, concomitant knockdown of p53 and MDM2 does not lead to further decrease of motility in most cases (there is one exception – cell line 1.21). Conversely, double knockdown seems to result in a slight increase of motility in some of the cell lines (A498), therefore it is likely that MDM2, rather than p53 is directly involved in promoting cell motility. However, it remains unclear whether

p53 also promotes motility in an MDM2-independent manner. To address this issue, p53 was knocked down in the 2.26 (117-derivative) cell line. In 2.26 cells, MDM2 expression is driven by the CMV promoter, therefore p53 depletion should not alter the level of MDM2. Subsequently, Boyden chamber motility assays were performed to determine whether reduction of the p53 level has any impact on motility while the levels of MDM2 remain intact. Western blotting was also performed to ensure that knockdown of p53 has no impact on the levels of MDM2 in this system.

This experiment shows that an efficient reduction of the level of p53 leads to neither reduction of motility nor reduction of the levels of ectopically expressed MDM2 which suggests that p53 on its own may not have any influence on cell motility in RCC as Figure 3.19 illustrates.

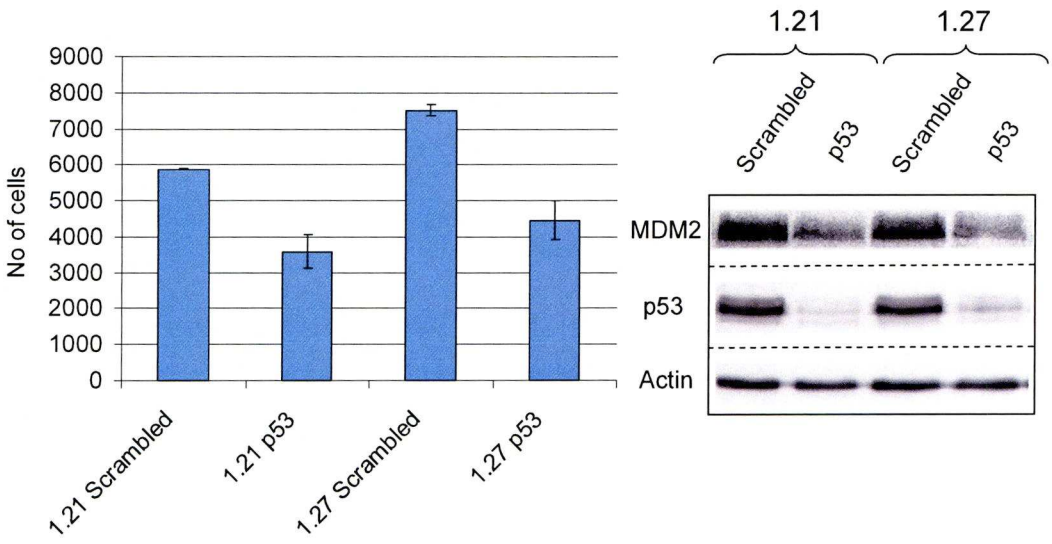


Figure 3.17 **siRNA mediated reduction of the p53 levels in "double high", highly motile RCC cells results in decreased MDM2 levels and motility.** The Figure shows data from a Boyden chamber motility assay together with western blot analysis to determine knockdown efficiency in 117 derivative cell lines 1.21 and 1.27. Cells which were transfected with 40nM scrambled siRNA or p53 siRNA using Lipofectamine 2000 as a transfection reagent and the motility assay was performed essentially as described in the legend for the Figure 3.16.

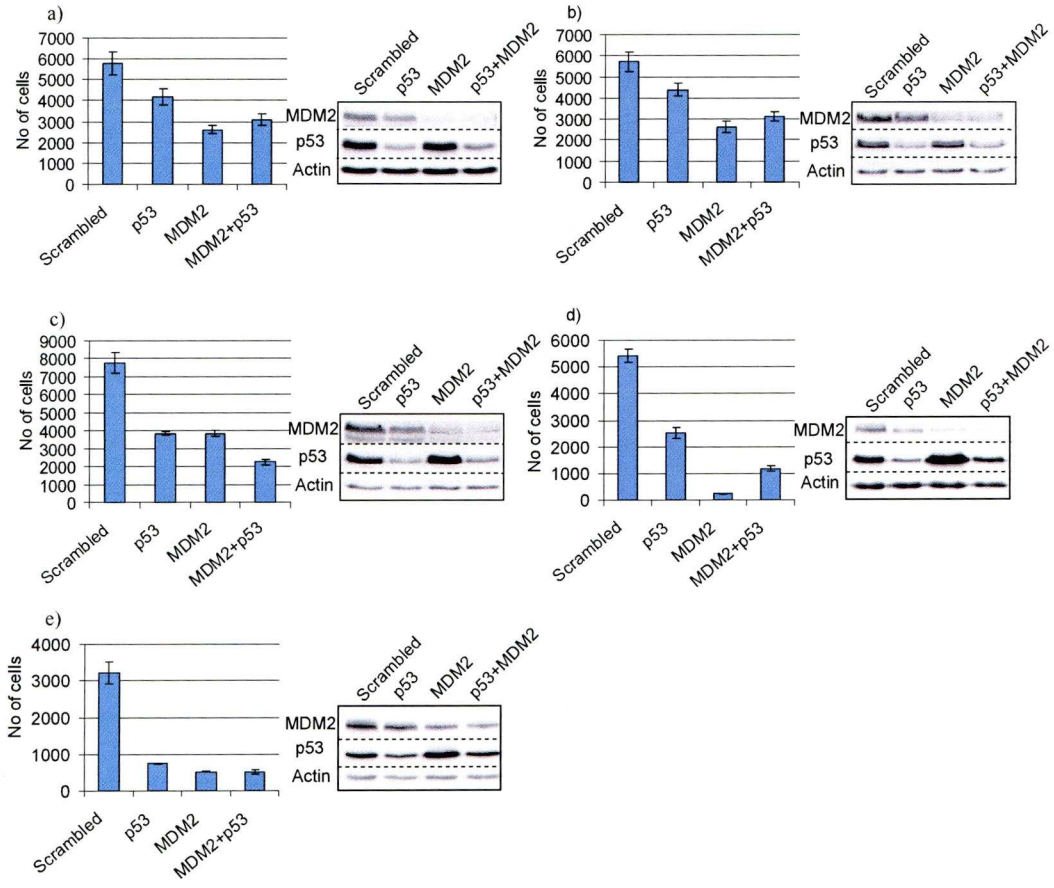


Figure 3.18 **Depletion of p53 and MDM2 in "double high" RCC cells.** The Figure shows the Boyden chamber motility assay, on the p53/MDM2-siRNA treated samples (as indicated) along with western blot analysis of 1.11 (*a*), 1.21 (*b*), 1.27 (*c*), A498 (d) and Caki-2 (e). Histograms show analysis of Boyden chamber motility assays performed essentially as described in the legend for the Figure 3.17 except, that 15,000 of A498 and Caki-2 cells was seeded into chambers.

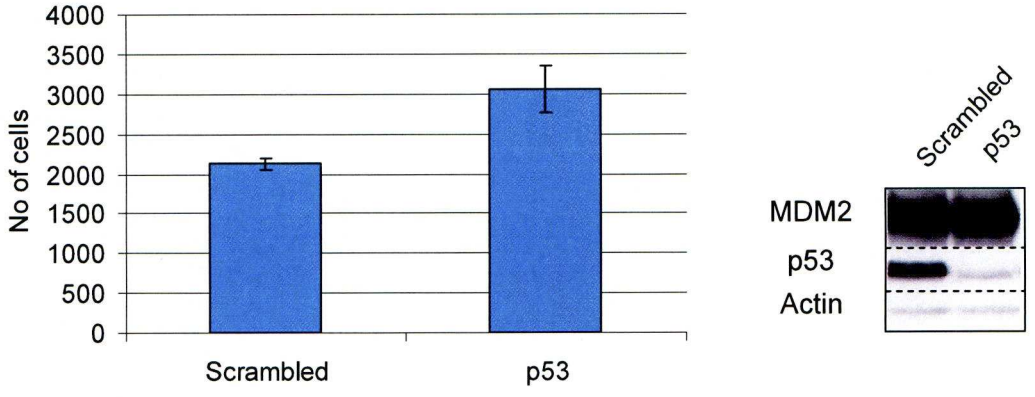


Figure 3.19 **Reduction of the p53 levels in 2.26 cells using siRNA.** The Figure shows the Boyden chamber motility assay and western blots control of the 117 derivative cell lines 1.21 and 1.27 performed essentially as described in the legend for the Figure 3.17.

3.3 p53 in RCC is wild type, functional and regulated by MDM2

Data presented in previous Section 3.2 strongly suggested that p53 is wt and functional in 117 derivative cell lines that have spontaneously acquired high levels of both p53 and MDM2 (A498 and Caki-2 were already known to harbour wt p53, data available from IARC database) as reducing p53 expression with siRNA led to reduction of the levels of MDM2. However, based on already published evidence, it was not clear, whether up-regulated p53 in RCC is usually wt or mutated (see Section 4.7), therefore, the status of p53 was examined in a set of tumour samples. The tumour samples were selected based on the expression of p53 and MDM2. 9 specimens which were positively stained (based on IHC performed by the members of our group) for both p53 and MDM2 were chosen to study the status of p53 in double high RCC tumours. Additionally, as it was suspected that specimens positively stained for only p53 might carry mutation in the *p53* gene (as mutated p53 would not be unable to contribute to over-expression of MDM2), 3 samples over-expressing only p53 were chosen. Since the IHC analysis identified also tumours in which only MDM2 is up-regulated, also 3 specimens positively stained for only MDM2 were added to the experiment. The FASAY technique (described in section 2.12), which is a yeast based technique allowing detection of mutated p53 alleles even from very heterogeneous samples (cancer cells may sometimes constitute a relatively small percentage of all cells in the biopsy), was used for analysis of these tumour samples. Out of these 15 tumour samples, only two samples carried missense mutations of p53 and both of these were present in the p53 and MDM2 over-expressing group. In one sample a G733T mutation was present, resulting in a G245C amino acid substitution. The second p53 mutated sample contained a C406G nucleotide substitution resulting in a Q136E substitution in the amino acid sequence.

All other samples were p53 wt which was confirmed by sequencing of 2-4 red colonies for each sample (for sequencing results see the Appendix 1). As a large number of PCR cycles were performed to amplify cDNA for recombination during FASAY, polymerase errors occur and contribute to the background of about 5% (5% red colonies in p53 wt samples). Considering a very low ratio of red to white colonies which in these samples did not exceed 5% (for comparison, the two samples in which mutations have been identified, contained nearly exclusively red colonies), and that the PCR errors are very unlikely to be introduced in the same site, it was assumed that the p53 status is wt unless the same mutation occurs in more than one clone.

The 117 derivative cell lines (see 2.21) were also tested using FASAY. No p53 mutations were detected in the 117 derivative cell lines. The analysis of the p53 status performed in both tumour tissue and cell lines suggest, that p53 is generally wt in RCC.

To test if wt p53 is functional in RCC cell lines (some evidence was already provided in experiments 3.2.1 and 3.2.2 which show that expression of MDM2 is p53-dependent) and responds to genotoxic stress; 117 and 1.27 cells were exposed to $4J/m^2$, $10J/m^2$, or $20J/m^2$ of UV-C light. As shown in Figure 3.20, UV treatment leads to stabilization of p53 in 117 cells which is in contrast with 1.27 cell line where no further stabilization of p53 can be observed, perhaps, because it is already stabile. Expression of p53 downstream genes appears to be variable; although induction of MDM2 and Bax in response to UV appears to be stronger in 117 than in 1.27 compared to the basal protein levels in these cell lines, highly induced levels of Bax and MDM2 in 117 cells seem to be comparable with the levels of non-induced proteins in 1.27 cells. p21 also becomes induced in 117 cells, contrasting with its

decrease in 1.27 in response to UV radiation. It can therefore generally be concluded, that p53 is functional and responds to stress (DNA damage) in both cell lines.

Since it was not clear what causes stabilization of p53 (it could be assumed that high levels of p53 could result from an inability of MDM2 to regulate p53 levels), the ability of MDM2 to regulate the levels of p53 in RCC cells was examined. To accomplish this, nutlin-3 (a small molecule that inhibits p53-MDM2 interaction) was titrated onto 117 and 1.27 cell lines in order to determine to what extent is p53 regulated by MDM2 in these cells. The experiment revealed that nutlin-3 treatment, and therefore inhibition of the p53-MDM2 interaction, results in a subtle accumulation of p53 in both 117 and 1.27 cells (Figure 3.21). On the other hand, MDM2 becomes strongly induced in both cell lines, and in 1.27 cells this process seems to occur even more efficiently. p21 also becomes induced in both 117 and 1.27, but to a much lesser extent. The results suggests, that increased levels of p53 and MDM2 in 1.27 cells can not be simply explained by the inability of MDM2 to degrade/inhibit p53, as MDM2 still appears to play a role in regulation of the p53 level and activity.

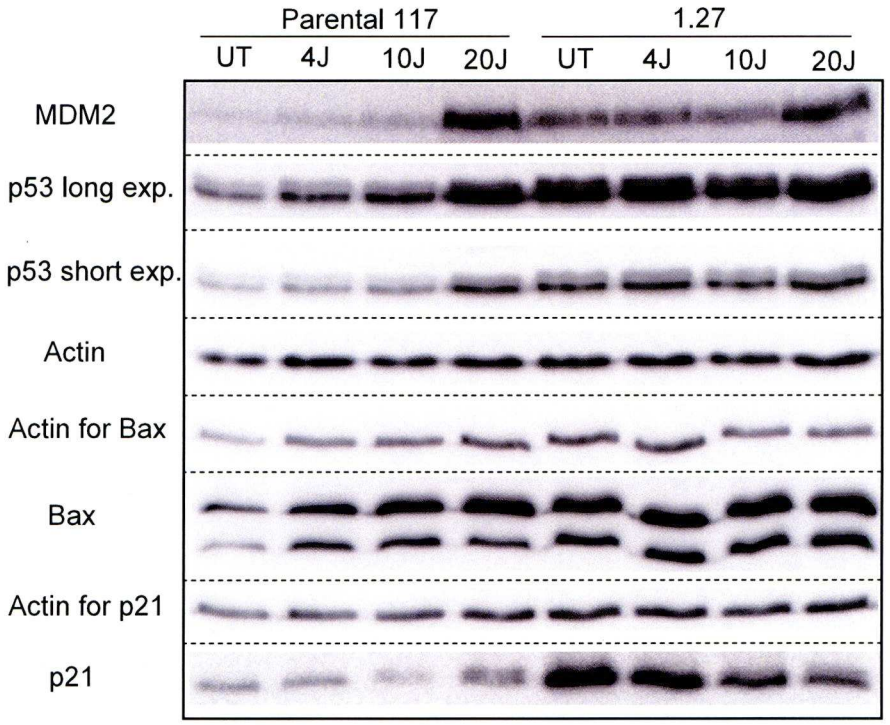


Figure 3.20 **DNA damage induces the p53 response in RCC cell lines.** 117 cells expressing low levels of p53 and MDM2 and 1.27 cells over-expressing p53 and MDM2 were treated with increasing doses of UV-C radiation as indicated. 24h after exposure to UV, the cells were harvested, and the protein extracts were subject to western blotting which shows expression of p53 and various p53-responsive genes: *MDM2*, *BAX* and *p21*. Actin was used as a loading control.

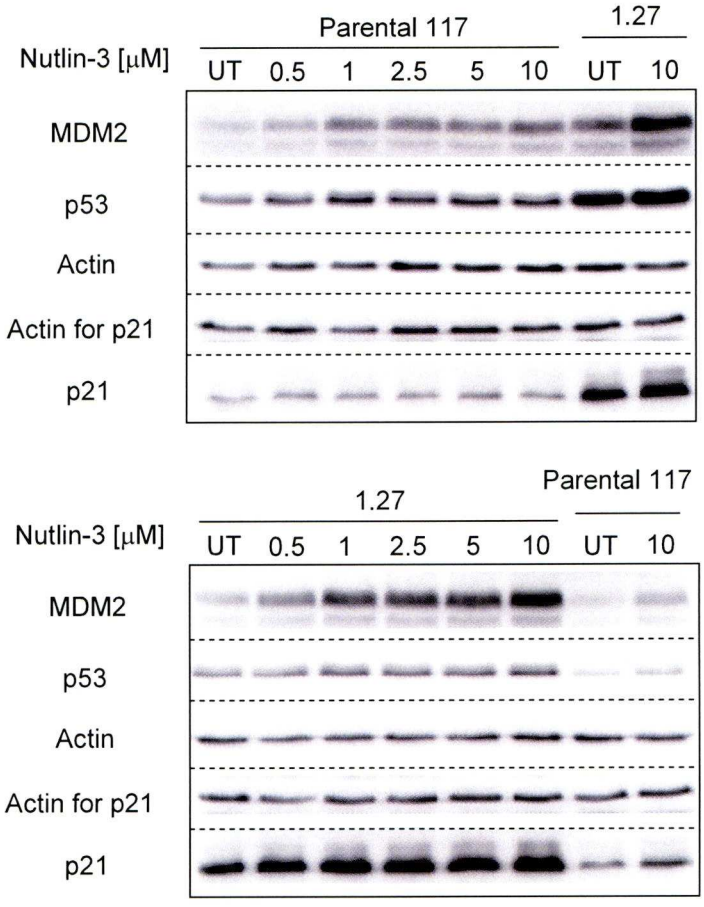


Figure 3.21 **Response of 117 and 1.27 cell lines to nutlin-3 treatment.** 117 cells expressing low levels of p53 and MDM2 and 1.27 cells over-expressing p53 and MDM2 were seeded and after 24h treated with increasing doses of nutlin-3 as indicated. After 24h cells were harvested, and the protein extracts were subject to western blotting which shows expression of p53 its responsive genes: *MDM2* and *p21*. Actin was used as a loading control.

3.4 Comparison of growth rate of 117 derivative cell lines

Since, as shown in previous sections, there is an obvious difference in the levels and activity of p53 between the "double high" and "double low" cells it could be assumed that p53 and/or MDM2 may alter proliferation in these cells. We therefore set out to investigate whether the difference in the p53 and MDM2 levels corresponds to any difference in proliferation. To address this issue, cell proliferation was measured.

The plotted growth curves show that high levels of p53 and MDM2 do not seem to have any obvious effect on cell proliferation, as shown in the Figure 3.22. This result also suggests that increased motility of "double high" cells obtained in the Boyden chamber motility assay cannot be due to any increased proliferation rate of these cells.

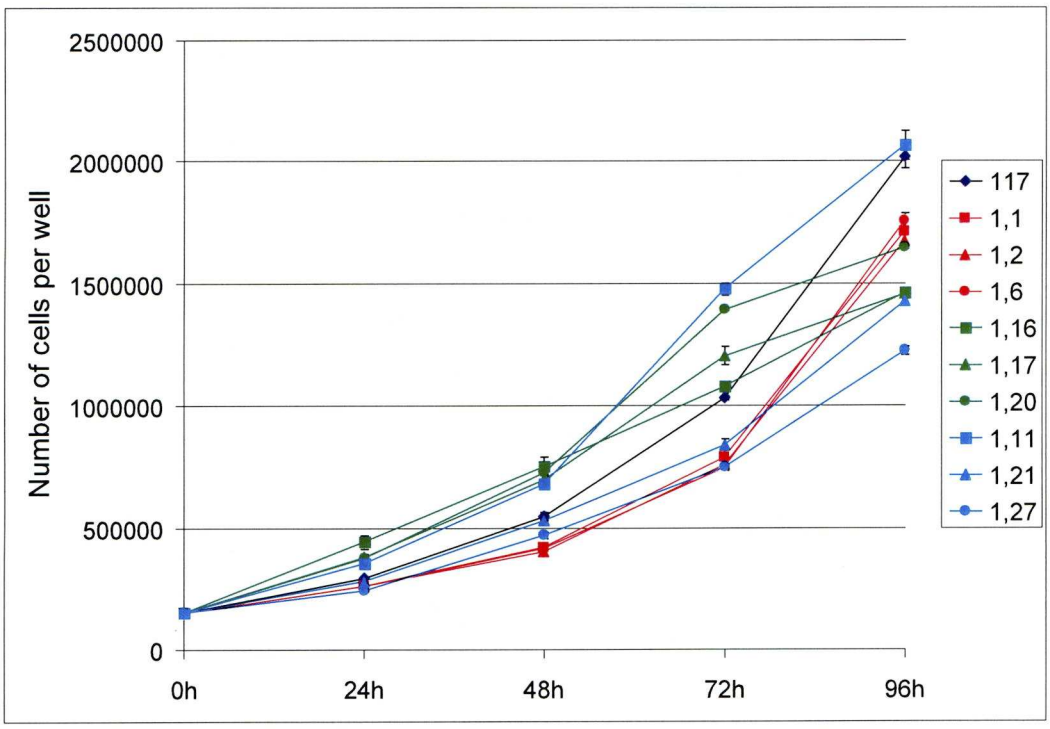


Figure 3.22 **Over-expression p53 and MDM2 in RCC cells does not affect proliferation.** 150,000 cells of each cell line was seeded per one well of the 6-well plate. Subsequently, cells growing in well (for each cell line) were harvested every 24h and counted. The numbers of cells counted at each time point for each cell line are presented on the histogram as the mean of three replicates +/- s.e.m. The red colour represents growth curves of "double low" cells, green represents "double intermediate" and blue represents "double high". The growth curve of the parental 117 cell line is presented in black.

3.5 High levels of MDM2 abrogate NME2-dependent motility suppression in RCC cells

NME2 proteins have a well-documented involvement in motility/metastasis suppression as mentioned before in Section 3.4 (this will be more extensively described and discussed in Section 4). Since MDM2 appeared to play a role in promoting motility of RCC cells (see Section 3.2), the functional aspects of NME2-MDM2 interplay with respect to motility were investigated. Expression of NME2, MDM2 and both proteins together were reduced using siRNA in 117 and 1.27 cells and cell motility was studied using the Boyden chamber assay.

The results revealed that NME2 functions in different ways in 117 and 1.27 cells. Although depletion of NME2 expression in 117 cells leads to increased motility, reduction of the MDM2 expression levels using RNAi, does not result in reduction of motility suggesting that low levels of MDM2 do not play a major role in the regulation of motility in these cells. On the other hand, treatment of highly motile 1.27 cells with NME2 siRNA does not result in increased motility, unless the levels of MDM2 are reduced with siRNA (Figure 3.23). This suggests that NME2 and MDM2 have opposite functions in regulation of motility and, perhaps, high levels of MDM2 block the motility-suppressive activity of NME2.

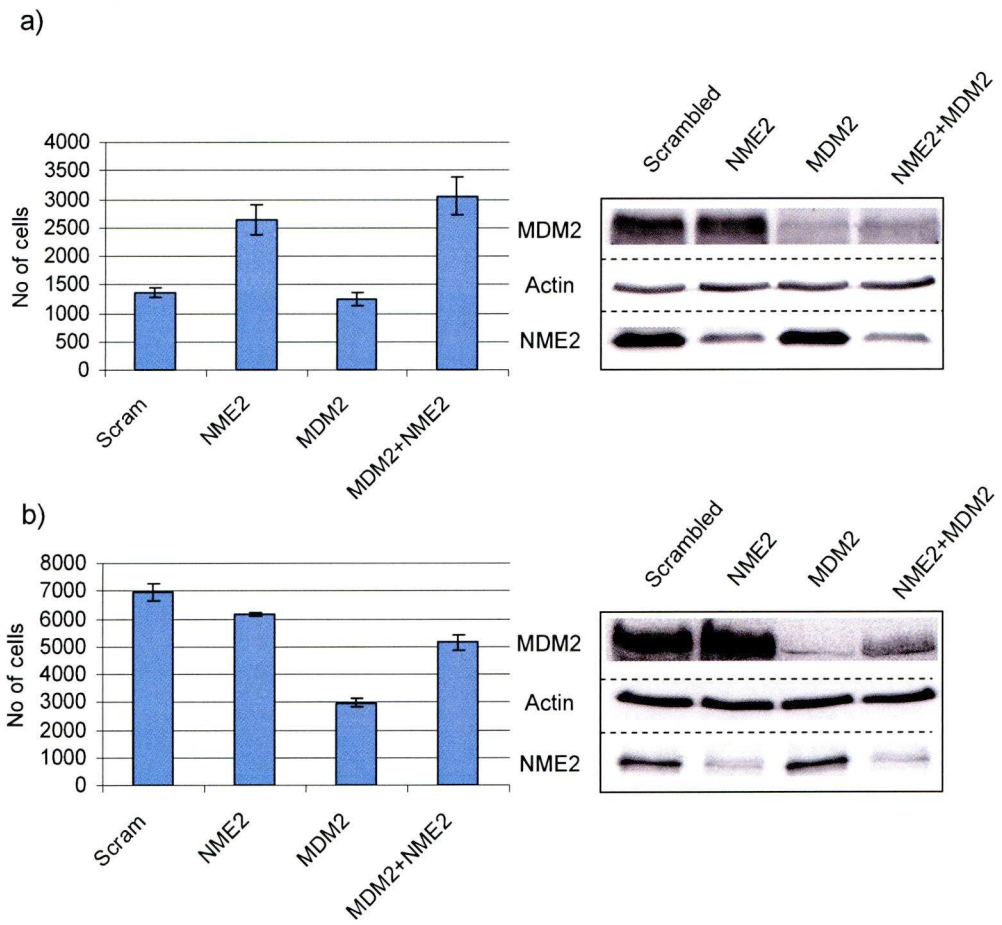


Figure 3.23 **MDM2 has an opposing effect on the NME2-dependent motility suppression.** The Figure shows the Boyden chamber motility assay performed on the siRNA-treated cells (as indicated) and western blots performed essentially as described in the legend for the Figure 3.17. The panel (a) represents the parental 117, and the panel (b) the 1.27 cell line.

4 Discussion

4.1 Introduction

Over-expression of MDM2 is associated with poor outcome in RCC (Haitel et al., 2000). Because this phenomenon is not a result of MDM2-dependent p53 degradation (p53 is usually also upregulated in MDM2 positive renal tumours), it suggests the existence of some alternative, MDM2-associated mechanism responsible for an aggressive phenotype of kidney cancers which express high levels of MDM2. The aim of this study was to identify MDM2 interacting proteins which could be involved in mediating poor outcome in RCC patients.

The yeast two-hybrid system allows detection of interactions between two proteins that are fused to either the DNA binding or activation domain of the Gal4 transcription factor (Fields and Song, 1989). Interaction is manifested by activation of marker genes that enable the auxotrophic yeast cells to grow on selective media and reporter genes which allow evaluation of the strength of the interaction. The yeast two-hybrid method, unlike various *in vitro* techniques gives a chance of investigating proteins synthesized and folded in natural conditions of the eukaryotic cell.

In this study, the yeast two-hybrid system was used to look for novel MDM2 interacting proteins expressed in RCC cells. For this purpose, a cDNA library was constructed using mRNA extracted from the RCC 786-0 cell line. This particular cell line was chosen as a source of mRNA because it was expected to reflect the situation present in a subset of in RCC tumours, which are characterised by highly aggressive phenotype associated with over-expression of both MDM2 and (supposedly) mutated p53. The use of mRNA from this cell line was intended to increase the chance of

finding the relevant MDM2-interacting proteins (the hypothetical interaction most likely takes place in such cells as they presumably manifest the phenotype common to the most aggressive RCCs. Subsequently, following transformation into yeast the library was then screened for interaction with MDM2. The screen reconfirmed previously described interactions of MDM2 with: ribosomal proteins L5, S7, L11, L26 and a transcription factor E2F1 (Elenbaas et al., 1996, Chen et al., 2007, Zhang et al., 2003, Ofir-Rosenfeld et al., 2008, Zhang et al., 2005). Several new potential targets were also identified. Of these, the candidates for MDM2 binding proteins which would be further investigated had to be selected. The NME2 protein appeared to be a good candidate for further study for a number of reasons. Firstly, it was also identified in our laboratory as a MDM2-interacting protein using a proteomic approach and thus two independent approaches provided evidence of an interaction. Secondly, NME2 has been identified as the PuF transcription factor and has been shown to contribute to expression of c-Myc (Postel et al., 1993), thus playing a role in regulation of expression of a potent proto-oncogene and, potentially also other genes involved in tumourigenesis. Finally, NME2 has been shown to function as a metastasis suppressor and therefore, hypothetically, its inhibition by MDM2 could explain how high levels of MDM2 increase aggressiveness of RCC. Therefore, NME2 protein became a subject to further analysis.

4.2 Authenticity and specificity of the NME2-MDM2 interaction

At the time, when the putative MDM2 interacting proteins identified in the screen were selected for further investigation, it was not obvious that both NME2 clones were not recombined with the pGADT7-Rec vector in a correct reading frame (as subsequently revealed by sequencing) and were thus potentially unable to produce

mRNA encoding the NME2 protein. Several lines of evidence support the conclusion that this out of frame fusion nevertheless produced NME2 protein fused in frame with the Gal4 AD and thus gave rise to yeast growth through interaction with MDM2. Firstly, the apparent interaction depended upon the presence of the cDNA since no interaction was detectable in the absence of this (when the vector expressing the AD only was used). Secondly, examination of this cDNA demonstrated that read-through from the Gal4 AD would produce a peptide of only 2 amino acids. It seems unlikely that such a small peptide expressed at the carboxy-terminus of a much larger fusion partner (Gal4 AD) could mediate the interaction that we detected. Thirdly, subsequent analysis of the full length NME2 cDNA expressed in-frame with the Gal4 AD confirmed that NME2 interacts with MDM2 in this system. In addition, several lines of evidence suggest that such an out of frame construct may still produce a protein of a correct amino acid sequence. It may occur at the level of transcription, when the RNA polymerase slips over a poly-adenine tract adding or losing 1 adenine nucleotide at a frequency that may reach 10% in mammalian cells (Linton et al., 1997, Benson et al., 2004). This mechanism appears to be conserved as a similar phenomenon has also been described in bacteria (Baranov et al., 2005). Interestingly, various studies show, that some viruses use polymerase slippage over homo-nucleotide tracts, to generate alternative reading frames from the same nucleotide sequence (Ratinier et al., 2008). A heptameric stretch of adenine is present in the pGADT7-Rec vector at the end of the Gal4 activation domain. Slippage at this site, resulting in the addition of one adenine, would lead to mutation of 2-3 codons (depending on the site of slippage) of the 3' terminus of the activation domain of Gal4 and synchronisation with a reading frame of downstream NME2 (see Figure 4.1). In this case, however, three STOP codons would appear in the 80bp-long spacer

between the Gal4 and NME2 ORF. Although several reports suggest that the STOP codon read-through events occur in yeast with frequency up to 3% (Williams et al., 2004, Namy et al., 2001, Mottagui-Tabar et al., 1998), the likelihood of successful read-through all three STOP codons present in the spacer between the Gal4AD and NME2 ORF is low. However, frameshift mutations may also occur at the translational levels. If such mutations occurred within the sequence of the vector between the last of the three STOP codons present in the sequence of the vector (see Figure 4.1) and NME2 ORF, then expression of NME2 would be possible. Although this event is extremely rare and appears with frequency of less than once in 100,000 codons (Farabaugh and Bjork, 1999), shortages of aminoacyl-tRNA may promote frameshifting by near-cognate decoding (O'Connor, 1998). As there is a shortage of amino acids present during selection of mated yeast on the plates, it is therefore possible, that this process might also contribute to the expression of NME2 in this study. This would result in the Gal4AD-NME2 fusion being less abundant; however, this could provide a growth advantage to the yeast cells, as studies of full length NME2 that was cloned in a correct reading frame into the pGADT7-Rec vector, showed that this protein appeared to be toxic to the cells. This toxicity was manifested by substantial inhibition of growth and irregularities in shape of the yeast colonies which contrasted with the original NME2 clones identified in the screen (the colonies grew robustly and were round).

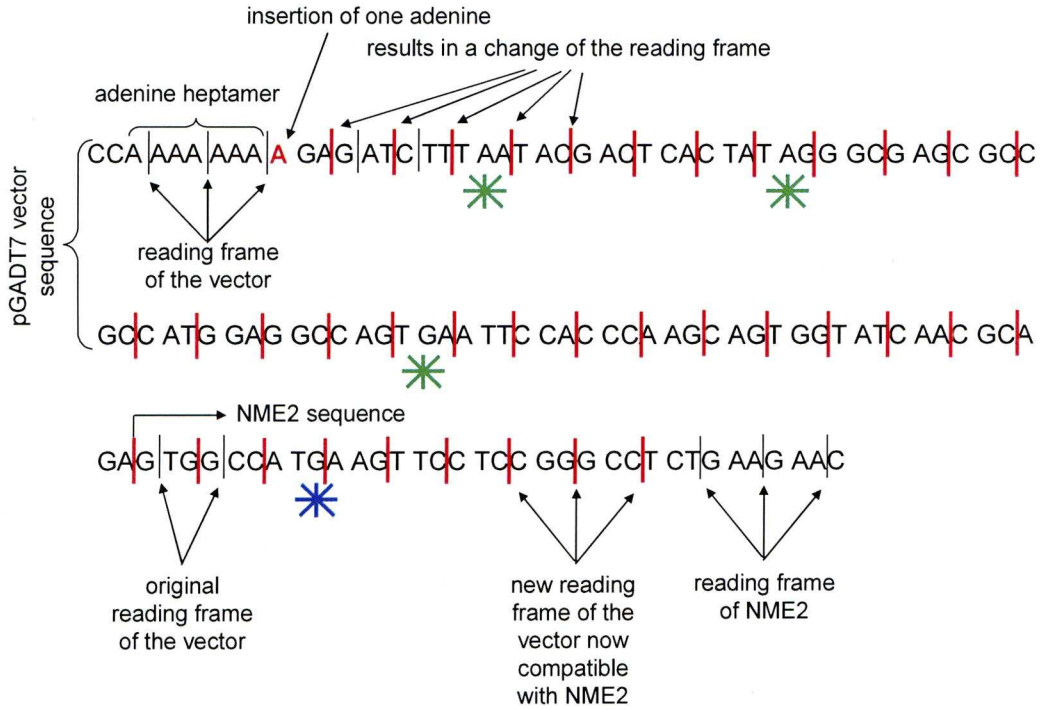


Figure 4.1 **Nucleotide sequence of the recombination site of the 5' end of the NME2 cDNA and the pGADT7-Rec vector.** The figure shows the nucleotide sequence of the pGADT7-Rec vector recombined with the incomplete cDNA (missing the 5' part) encoding the NME2 protein. The sequence is divided into coding triplets as indicated. Insertion of an additional adenosine (red colour font) due to polymerase slippage results in a shift of the reading frame (as indicated by red lines) and accordance with the reading frame of NME2. Green stars indicate STOP codons created as a result of the polymerase slippage and the blue star indicates the STOP codon terminating expression of the peptide encoded by the out of frame NME2 cDNA (when no slippage occurs).

When it became apparent that NME2 clones identified in the yeast two-hybrid screen were not recombined with the expression vector in a correct reading frame, the cDNA encoding the full length NME2 was cloned into the pGADT7 vector and the interaction was retested with a positive result (see 3.1.4 for details). However NME1, which shares 88% of amino acid identity and a very high degree of homology across the remaining sequence with NME2 (see Figure 4.2), did not detectably interact with MDM2 in yeast. This suggests a very high specificity of the interaction and as will be discussed later in this section, this difference may determine the ability of NME proteins to regulate the levels of MDM2.

```
NME1  N'MANCERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVGLKFMQASEDLLKEHYVDLKDRPFFAGLVKYMHSGPVVAMV

NME2  N'MANLERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQHYIDLKDRPFFPGLVKYMNSGPVVAMV
        *** ********************************.:**::***: **:**:********.******:********

NME1   WEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSVESAEKEIGLWFHPEELVDYTSCAQNWIYE C'

NME2   WEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSVKSAEKEISLWFKPEELVDYKSCAHDWVYE C'

       *********************************************:******.***:*******.***::*:**
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Figure 4.2 **Clustal W (1.83) alignment of NME1 and NME2 amino acid sequences.** The Figure shows a comparison of the amino acid sequences of NME1 (gi|49457226) and NME2 (gi|49456397). "*" refers to amino acids that are identical in given positions, ":" indicates conservative substitutions, "." indicates semi-conservative substitutions and gaps refer to non-conservative substitutions.

4.3 NME proteins have a variety of different functions

Proteins from the NME family, also called NM23 (non-metastatic 23) or NDPK (nucleoside diphosphate kinase) are well known for their nucleoside diphosphate kinase activity. However, several lines of evidence suggest that in addition to their nucleoside diphosphate kinase activity ((EC 2.7.4.6, which is responsible for the generation of nucleoside triphosphates from nucleoside diphosphates (Otero, 2000), NME proteins have also different properties and indeed, the list of other functions of the NME proteins is a long one. NME2 has been identified as the PuF transcription factor which has been shown to contribute to expression of c-MYC (Postel et al., 1993). In addition, it has been demonstrated that an ancestral form of human NME proteins (called AWD in *Drosophila* which has only one homologue) is essential for drosophila development, since null mutations caused larval lethality, suggesting that the activity of NME proteins may also be essential for development in higher animals (Xu et al., 1996). There are 8 *NME* orthologs in mouse and human and *Nme1* is the only member of the *Nme* gene family that has been knocked out in higher animals. Analysis of *Nme1*-null mice has demonstrated that Nme1 protein is not essential for life. Mice lacking *Nme1* displayed growth retardation and a lactation disorder, nevertheless, *Nme1*-null animals otherwise develop normally (Arnaud-Dabernat et al., 2003), most likely due to high functional redundancy between the members of the NME protein family. However, analysis of a hepatocellular carcinoma model on an *Nme1* KO background mice revealed that the animals suffered from a higher incidence of pulmonary metastasis, thus suggesting (along with other evidence described in Section 4.5) a role for *Nme1* in metastasis suppression (Boissan et al., 2005). Interestingly, tumour cells from the *Nme1* KO mice expressed higher levels of Nme2, compared to matched normal tissues. This suggests the existence of a

feedback mechanism leading to compensation of Nme1 function by the Nme2 protein. However, it also shows that at least some anti-metastatic functions of Nme1 and Nme2 are not redundant as *Nme1* KO mice develop more metastases despite the potential compensation provided by up-regulation of Nme2 (Boissan et al., 2005).

As described above, NME proteins play various roles in cells. In the present study, additional activities of NME2 were identified. NME2 was shown to regulate the transcriptional activity of p53 and also to regulate the steady state levels of MDM2. The data also suggest, that NME2 may play a role in regulation of the post-translational modifications of p53, thus, further expanding the potential functional repertoire of NME proteins.

4.4 NME2 and NME1 suppress the activity of p53

Considering the pleiotropic nature of NME proteins (see above), it was difficult to predict what would be the consequence of the interaction between MDM2 and NME2. First, it was decided to investigate if NME2 has any influence on MDM2, because the appropriate techniques were well optimised and routinely used in our laboratory. The most sensitive method of detecting changes in the activity of MDM2, and which was available in our laboratory, was to perform a luciferase assay to measure the activity of p53. The assay was therefore used to test if the NME2-MDM2 interaction had any effect on the activity of MDM2 towards p53 (which would be reflected by changes in the activity of p53). Experiments performed in tissue culture as shown in Section 3.1.4, revealed an activity of NME2 that could regulate p53 activity. Over-expression of NME2 in human cancer cell lines H1299 and MCF-7, led to a reduction in the activity of the ectopically expressed p53 protein (Figures 3.7, 3.8). Moreover, the kinase activity of NME2 appears to be dispensable

for this effect (Figure 3.10) and the observed reduction of p53 activity was not accompanied by a reduction in the p53 protein level. This latter suggests that the effect is on the intrinsic activity of p53 and not on the steady state levels of the protein. The experiments performed in normal cells, BJ fibroblasts (Figure 3.9, 3.11) further supported the data from cancer cell lines and provided evidence, that NME1 also reduces the activity of p53 in a similar way to NME2. Moreover, transfection experiments using double null (*Mdm2*$^{-/-}$, *p53*$^{-/-}$) MEFs revealed that NME2 and NME1 inhibit the activity of p53 independently of MDM2. These results do not accord with some data that were published while this project was being carried out (Jung et al., 2007). On the basis of similar experimental procedures to these used in the present study, these authors came to the conclusion that NME1 interacts with p53 and induces its activity. The panels of cell lines used by Jung et al., overlaps with the ones used here (MCF-7 and H1299), however, the authors did not use any non-cancer cells (such as BJ fibroblasts used in the present study) or cells providing an *Mdm2*-null background (double-null MEFs) to validate their results. Thus the results presented in the current study are more reliable.

4.5 NME2 alters the MDM2 dependent modification of p53 and decreases the levels of MDM2

Analysis of western blotting (Figures 3.7, 3.8) suggested that not only the activity, but also the MDM2-dependent post-translational modifications of p53 might be regulated by NME2. MDM2 is known to promote post-translational modifications such as ubiquitylation, sumoylation or neddylation of several target proteins including itself and p53 (Honda and Yasuda, 2000, Xirodimas et al., 2004, Meek and

Knippschild, 2003, Melchior and Hengst, 2002), often with profound effects on stability and activity of the target protein. The results presented in this thesis show that the ladder of high molecular weight forms of p53 visible above the main un-modified form of the protein, present in samples transfected with p53 and MDM2, seems to disappear after co-transfection with NME2. The molecular weight of the second p53 immunoreactive band has an apparent MW of 60kDa which corresponds to the expected apparent MW of mono-ubiquitylated p53 (Figures 3.7, 3.8 and 3.14). More interestingly, co-transfection of NME2 appears to change the profile of the ladder and seems to promote enrichment of the mono-ubiquitylated fraction (Figure 3.14). A RING finger mutant of MDM2 C464A does not promote any detectable modification of p53 which suggests that the p53 ladder, present in the samples co-transfected with MDM2, is produced by an activity of the RING-domain of MDM2 (Figure 3.14). Since the RING-domain of MDM2 carries the E3 ubiquitin ligase activity, one could speculate that the ladder is composed of mono- and poly-ubiquitylated p53 molecules (as it has been shown that the RING finger of MDM2 promotes NEDDylation of p53 (Xirodimas et al., 2004). In such a scenario one could hypothesize that the NME2 protein may also take part in regulation of p53 levels (if the ladder is composed of poly-ubiquitylated forms of p53) independently of its ability to regulate the activity of p53. Figure 3.14 supports this notion as coexpression of NME2 with p53 and MDM2 seems to increase the intensity of the band corresponding to the main (un-modified form) of p53. This does not appear to happen in experiments where much smaller amounts (for details see Section 2.18) and a higher ratio of p53 to MDM2 plasmid DNA were used (see Figures 3.7 and 3.8). This could be explained by the fact that the amount of p53 present in the ladder in these latter experiments (Figures 3.7 and 3.8) is relatively insignificant compared

with the amount of unmodified p53 present in the main band, most likely due to low levels of transfected MDM2 which do not promote efficient modification of p53 (the ratio of transfected p53 to MDM2 vectors was 1:3 in these experiments). Hence the effect of NME2 on the unmodified fraction of p53 is masked by the high basal level of p53. On the other hand, large amounts of plasmid DNA and a lower ratio of transfected p53 to MDM2 (1 : 6) as used in the experiment described in Section 3.1.7 (Figure 3.14) significantly increased the amount of p53 present in the ladder fraction, but also decreased the relative intensity of the main p53 band. This allows detection of the NME2-mediated changes of the intensity the main band of p53 (as well as the ladder) in this experiment.

Western blot analyses of MDM2 levels after co-transfection with NME2 suggest the possibility that NME2 is involved in the regulation of MDM2 levels as the MDM2 band appears less intense in samples that were co-transfected with NME2 (Figures 3.7 and 3.8). The effect seems to be much more pronounced with larger amounts of the plasmid encoding MDM2 used for transfection and is independent of any other known function of NME2, as either the H118F or K12Q mutants appear to function similarly with respect to MDM2 (Figures 3.12, 3.13, 3.14). Interestingly, co-overexpressed NME1 does not lead to reduction of the MDM2 levels (Figure 3.13), suggesting a high specificity of the NME2-mediated effect on MDM2. Further investigation of this phenomenon revealed that the NME2-dependent downregulation of MDM2 can be blocked using the proteasome inhibitor MG132, indicating that 26S proteasomes are essential for this process (Figure 3.13). Even more interestingly, the C464A RING-finger mutant of MDM2 appeared to be refractory to NME2-dependent downregulation, which suggests that the effect is dependent on an intact RING-domain of MDM2. Moreover, this effect appeared to be mediated by the N-

terminus of NME2, as the chimeric protein in which the N-terminus of NME2 was replaced with the N-terminus of NME1, failed to reduce the level of MDM2 (Figure 3.15). One can therefore hypothesise that NME2 increases auto-ubiquitylation and, as a consequence, proteasomal degradation of MDM2. The ability of NME2 to decrease the levels of MDM2 could also explain why the intensity of the MDM2-mediated post-translational modification of p53 decreases in samples co-transfected with NME2, since it has been shown that low levels of MDM2 promote mono-ubiquitylation of p53 rather than poly-ubiquitylation (Li et al., 2003). This is consistent with a decrease of the intensity of the ladder in the fraction corresponding to poly-ubiquitylated p53 and enrichment of the mono-ubiquitylated forms of p53 in observed in samples co-transfected with NME2.

4.6 MDM2 promotes motility in RCC cells and opposes the ability of NME2 to suppress cell motility

Apart from involvement of NME2 in regulation of MDM2 levels, another potential connection between NME2 and MDM2 - regulation of motility was proposed and is going to be discussed later in this Section. It will, however, be preceded by discussion over the ability of MDM2 to promote motility of RCC cells (a feature that strongly links MDM2 with aggressive phenotype of cancer cells), which was also discovered in the present study.

One of the main goals of this project was to identify phenotypic changes that result from upregulation of MDM2. This would help the initial selection of potential MDM2 binding proteins for further investigation from those identified in the screen, with respect to their putative involvement in mediating the aggressive phenotype of

RCC. For this purpose, the isogenic clones of the UOK117 RCC cell line were obtained (see Section 2.21) and a panel of cell lines expressing high levels of both p53 and MDM2 (double high), low levels of both p53 and MDM2 (double low) or clones expressing intermediate (double intermediate) levels of p53 and MDM2 was selected (Figure 3.16a) and characterised with respect to motility and p53 status. As the cells that have spontaneously acquired expression of high levels of p53 and MDM2 have been obtained by selection and not by forced, ectopic expression of proteins, it was suggested, that this process could be a surrogate of the natural selection occurring in progressing RCC tumours and therefore serve as a model of RCC progression.

Subsequent analysis revealed dramatic changes in cell behaviour. Double high cells appeared to be about 10-fold more motile than the parental 117, double low and intermediate clones (Figure 3.16b). Moreover, siRNA-mediated reduction of the MDM2 expression levels resulted in inhibition of motility in highly motile double high cells. It has previously been suggested by others, that MDM2 may regulate cell motility via ubiquitylation and degradation of E-cadherin (Yang et al., 2006). It has been shown, that MDM2 upregulation correlates with decreased levels of E-cadherin in high grade breast tumour clinical samples. These results do not accord with ours as the experiments performed in our lab show that the RING-finger mutant of MDM2 (unable to promote ubiquitylation of E-cadherin), over-expressed in H1299 cells also promoted motility (Polanski et al. manuscript in preparation) suggesting that ubiquitylation and degradation of E-cadherin may not be the only way in which MDM2 influences motility.

Interestingly, depletion of p53 also led to decreased motility and this was associated with reduction of MDM2 levels (Figure 3.17). Importantly, reduction of the levels of

both p53 and MDM2, performed on a number of occasions in five individual double high cell lines did not lead to further reduction of motility (with only one exception, Figure 3.18). To exclude the MDM2-independent role of p53 in regulation of motility, an experiment involving the 2.26 cell line which expresses high levels of endogenous p53 and exogenous MDM2 was performed (Figure 3.19). The expression of MDM2 in 2.26 cells is driven by the CMV promoter; hence, is independent of p53. Although the experiment did not demonstrate that exogenously expressed MDM2 contributes to increased motility in these cells (which is two fold higher than in parental 117 or double low cells), it shows that depletion of p53 using siRNA transfection in the 2.26 cell line does not result in reduction of MDM2 levels and cell motility. This suggests that the reduction of motility in the p53/MDM2 siRNA experiments (see Section 3.2) was not caused by inhibition of MDM2-independent activity of p53.

Once the role of p53 and MDM2 as motility promoting agents in RCC cell lines was identified, the functional consequences of NME2 - MDM2 relationship with respect to cell motility were studied in RCC cell lines.

It has been demonstrated by others that NME1 and NME2 suppress cell motility and it has been suggested that both proteins function as metastasis suppressors (Rayner et al., 2008, Jung et al., 2006). NME2 has been reported to be expressed at higher levels in non-metastatic versus metastatic cells, for example oral squamous carcinoma cells. In addition, transfection of cells with NME2 has been shown to reduce their metastatic potential, which suggests a role of NME2 in metastasis suppression (Miyazaki et al., 1999). Similarly to NME2, NME1 has also been shown to be down-regulated in numerous metastatic types of cancers such as: melanoma,

neuroblastoma, breast, colorectal, liver, gastric, ovarian, prostate, cervical, thyroid, lung and pancreatic suggesting its function in metastasis suppression (reviewed in (de la Rosa et al., 1995).

Two mechanisms for NME1-mediated metastasis suppression have been proposed so far. Studies of a number of different carcinoma and cell lines have suggested the inverse correlation between the expression levels of NME1 and the lysophosphatidic acid receptor EDG2. Transfection experiments revealed, that NME1 downregulates EDG2 expression, thus reduces the EDG2-dependent motility and overexpression of EDG2 has been shown to rescue this effect (Horak et al., 2007a). A subsequent study has shown that restitution of EDG2 expression in NME1-overexpressing cell lines markedly increased their metastatic properties in nude mice (Horak et al., 2007b), further supporting the *in vitro* data.

It has also been demonstrated, that NME1 regulates the Rac1-dependent cell motility through binding and inhibition of Tiam1 (Rac1-specific nucleotide exchange factor) which leads to inhibition of the Rac1-GTP complex formation. Interestingly, this effect appears to be independent on the kinase activity of NME1 suggesting that, perhaps, the protein-protein interaction of NME1 with Tiam1 inhibits activation of Rac1 and the downstream JNK pathway (Otsuki et al., 2001).

No molecular mechanism for NME2-mediated motility/metastasis suppression has been proposed so far. It reflects the general tendency for NME2 being less studied than NME1. However, considering high homology between the two and reported involvement in regulation of the same processes (motility/metastasis suppression) it is not unreasonable to assume, that NME2 acts in a similar way to NME1.

In the present work, it was observed, that reduction of the levels of NME2, MDM2 and both proteins simultaneously using siRNA, appeared to have different

consequences in the parental 117 cells and double high 1.27 cells. In 117 cells, depletion of NME2 leads to induction of motility (consistently with previously published data) both in the presence and absence (siRNA treatment) of endogenous MDM2 (Figure 3.23a). However, in 1.27 cells which express high levels of MDM2, NME2 depletion does not lead to further induction of motility. Consistent with data presented previously (Figure 3.18), reduction of MDM2 levels, results in decreased motility. This could to be due to the action of NME2 (which now functions in absence of MDM2), since a double NME2+MDM2 siRNA-mediated reduction of protein expression partially rescues the decrease of motility caused by MDM2 depletion (Figure 3.23b). It can therefore be suggested that MDM2 and NME2 have opposing effect on motility, yet regulate different pathways modulating cell motility. Alternatively, the results could be interpreted as follows: high levels of MDM2 directly block the ability of NME2 to suppress cell motility (this could be a result of the protein-protein interaction) and either constitutively expressed low MDM2 levels or its high levels reduced using siRNA (in cell overexpressing MDM2) allows functioning of NME2.

The finding suggests a potential therapeutic strategy, which could be based on either reduction of MDM2 levels (if decreased levels of MDM2 allow NME2 to function) or prevention of the MDM2-mediated effect on NME2 (preventing the protein-protein interaction if this interaction results in inhibition of the NME2-mediated motility suppression) and potentially lead to inhibition of metastatic spread.

4.7 p53 is wt, functional and drives overexpression of MDM2 in RCC cells

Several additional issues were raised in this study as a consequence of investigating the p53 status and its relationship with MDM2 in RCC. It has previously been demonstrated, that p53 overexpression in various types of cancers such as oesophageal, breast, colorectal, lung or ovary usually indicates its mutation (Shi et al., 1999, Davidoff et al., 1991, Yang et al., 1999, Westra et al., 1993, Marks et al., 1991). Although some studies report that p53 mutations are very rare in RCC (Suzuki et al., 1992), the others present data suggesting that p53 is mutated at high frequency in sarcomatoid RCC (sarcomatoid RCC is a highly aggressive form of RCC characterised by the presence of tumour cells with morphology resembling various types of mesenchymal cells, such as miofibroblasts (Delahunt, 1999), (Oda et al., 1995).

Mutation of p53 in the p53-MDM2 overexpressing tumours was one of the assumptions leading to construction of the RCC tumour progression model based in 117-derivative cell lines (see Section 2.21). It was hypothesized that cells harbouring mutated and stabile (and therefore overexpressed) p53, somehow acquire high levels of MDM2. Also based on this hypothesis, the 786-0 RCC cell line, expressing mutated p53, was used for construction of the library (subsequently screened for the MDM2 binding proteins). The results described in the present study clarify the issue of the p53 status in RCC. To contradict the founding assumptions and the data from other carcinomas mentioned above, the results suggest that p53 is usually wt in p53 over-expressing RCC tumours, as only 2 samples out of 12 (p53 over-expressing) tested by FASAY, carried p53 mutation. Moreover, no 117-derivative cell line over-expressing both MDM2 and endogenous p53, harboured p53 mutation. Additionally,

direct sequencing of the genomic DNA sequence of the 786-0 cell line did not confirm presence of the expected 832C>G missense mutation resulting in the P>A amino acid substitution in the codon 278 (IARC TP53 mutation database).

This also suggests that the function of wt p53 must be compromised in RCC to allow cell growth and survival. It has been proposed before, that p53 is not functional in RCC cell lines (which express wt p53) and that the p53 pathway is repressed by an unidentified dominant mechanism (Gurova et al., 2004). The results obtained in our lab show, however, that p53 is functional in RCC cell lines and its levels are significantly regulated by MDM2 (Warburton et al., 2005). It would therefore be expected, that increased levels of MDM2 lead to reduction of p53 as a result of the MDM2-dependent ubiquitylation and degradation of p53 (Honda et al., 1997). However, it is shown here that although MDM2 still plays a role in regulation of the stability and activity of p53 (see Section 3.2), yet it is unable to reduce the level of p53 in double high cell lines. The results presented in this thesis suggest repression of the p53 pathway downstream from p53 rather than repression of p53 itself. It was demonstrated that p53 is functional and, although the "double high" cells have greatly increased levels and activity of p53, they neither die nor proliferate slower as a result of p53-dependent activation of genes such as *BAX* and *p21*.

The first evidence that p53 is functional in the cell lines used in this project, was provided in the Figure 3.18, where depletion of p53 using siRNA in the double high cells resulted in markedly decreased expression of MDM2 which did not happen when MDM2 was expressed ectopically from the CMV promoter (Figure 3.19). This suggests that high levels of MDM2 are promoted by high levels of p53 in cells which spontaneously acquired high levels of both proteins.

Additionally, the p53 functionality, as well as the ability of MDM2 to regulate the levels and activity of p53 was tested in the double high 1.27 and a parental 117 cell line. The UV-induced DNA damage led to strong stabilization of p53 in 117 and, even though further p53 stabilization did not occur in 1.27 cells, a significant induction of MDM2 could be seen in both cell lines (Figure 3.20). The inability of 1.27 cells to increase the p53 level upon DNA damage could be due to the presence of already stabile p53. However, elevated expression of MDM2, and to a much lesser extent also BAX, suggests that p53 could be further activated by UV in the 1.27 cell line. Moreover, the steady-state level of p21 is much higher in 1.27 than in parental 117 cells (Figures 3.20 and 3.21) suggesting, that elevated levels of p53 increase transactivation of the p21 gene. Interestingly, the Figure 3.20 shows that the p21 levels decrease in response to UV in 1.27 cells; this could be explained by general suppression of gene expression caused by UV. However, downregulation of p21 could potentially act as an element of apoptotic response of the cells (or an attempt), since it has been shown that downregulation or deletion of p21 markedly increases apoptotic rate in various types of cells (Yu et al., 2003, Park et al., 2008). This can further be supported by the fact, that no suppression of the p21 expression can be seen in parental 117 cells suggesting that this change may be specific and not caused by general suppression of gene expression after UV treatment.

It is unclear, to what extent is the p53-MDM2 negative feedback loop functional in RCC cells, but clearly MDM2 does not promote efficient degradation of p53. It was shown that titration of a small compound which blocks binding of MDM2 to p53, nutlin-3 (Vassilev et al., 2004), led to stabilization of p53 in 117 cells. No further stabilization could be seen in 1.27 cells, perhaps (as suggested previously), because p53 was already stabile in this cell line. This was accompanied by significant

induction of MDM2 and to a lesser extent also p21 in both 117 and 1.27 cell lines. These results suggest that, even though the p53 levels are elevated in some RCC cells (such as 1.27), p53 is still regulated by MDM2 in these cells as a specific inhibition of MDM2 results in activation of p53 and leads to increased expression of p53-downstream genes (Figure 3.21). These results clearly indicate that high levels of MDM2 are driven by elevated levels (and, perhaps also) activity of p53 in RCC cells given that the siRNA-mediated decrease of the level of p53 reduces both the MDM2 level and motility (which is a result of upregulation of MDM2). One could speculate that in RCC cells which over-express wt p53 and MDM2, p53 acts as an oncogene as it neither induces apoptosis nor decreases the cell proliferation rate (Figure 3.22). On the other hand, however, it drives overexpression of MDM2 which is responsible for increased motility and, perhaps, also other features contributing to poor outcome in RCC patients. It could therefore be speculated, that anticancer therapies relying on further activation of p53 (for instance commonly used chemotherapeutic drugs such as cysplatin) in RCC tumours harbouring high levels of p53 and MDM2, could not be effective as they would simultaneously result in further induction of MDM2 (see 3.21) and, perhaps, contribute to even worse outcome. Therefore, further studies addressing this issue are needed.

4.8 Future plans

As mentioned several times in this document, elevated levels of p53 and MDM2 increase tumour aggressiveness, for reasons which have not been explained to date. Previously published evidence described in the introduction, is restricted to analysis of the phenotype of the disease, based on clinical data. Results presented in this

thesis strongly suggest a role of MDM2 in promoting an aggressive phenotype of RCC cells. The study, however, raises several questions:

1. Is blocking of the motility suppressive effect of NME2 by MDM2 increasing aggressiveness of RCC cells in the real tumour?
2. Would it be the only way MDM2 functions to promote poor outcome?
3. Why are high levels of active p53 not mediating apoptosis or the cell cycle arrest? Is the ability of p53 to mediate apoptosis counterbalanced by high levels of MDM2?
4. Would further activation of p53 accompanied by downregulation of MDM2 be a good therapeutic strategy to target RCC overexpressing p53 and MDM2?

There are no definitive answers to these questions. The points 1 and 2 will be addressed in future studies using mouse xenograft experiments and further hunting for MDM2 interacting proteins. Questions 3 and 4 will also soon be investigated in our lab. Since RCC cells accumulate high levels of active p53, then, why do high levels of wild type and active p53 not induce apoptosis in RCC (it doesn't also appear to induce cell cycle arrest or senescence), like they would do in other types of cancers? On one hand, it has been suggested that this could be due to a natural propensity of renal cells to not respond by apoptosis to increased levels of p53 (MacCallum et al., 1996). The study has demonstrated that unlike other epithelia, such as the gut lining, tubular epithelial cells do not apoptose in response to IR despite increased levels of p53. However, this conclusion was drawn based on studies of cells exposed to ionising radiation which induces the DNA damage response (mice were treated with whole-body irradiation) which may not reflect the situation present in the tumour where the number of stresses that could potentially

activate p53, may be both higher and of different kinds. Interestingly, others have reported p53-dependent apoptosis of renal tubular epithelial cells (these cells give rise to RCC) in response to stimuli other than IR (Li et al., 2007, Choi et al., 2001, Kelly et al., 2003).

The results presented in this thesis suggest that blocking the MDM2 mediated inhibition of p53 using nutlin-3 does not appear to effectively induce apoptosis (data not shown), despite increased expression of p53 responsive genes. Perhaps this is due to the counterbalancing action of MDM2 which becomes very strongly induced upon nutlin-3 treatment (see Figure 3.21). Our unpublished (not presented here) observations suggest that reduction of MDM2 protein levels using siRNA, decreases the number of cells in culture. Therefore one could hypothesize that a therapeutic agent decreasing the level of MDM2 (and therefore, potentially inhibiting also survival or aggressiveness-promoting features of MDM2), applied together with chemotherapy (p53-inducing genotoxic agents such as cisplatin), would be a good way of targeting RCC overexpressing p53 and MDM2.

5 Conclusions

High levels of p53 and MDM2 in RCC tumours correlate with increased aggressiveness resulting in poor outcome in patients. The nature of the p53-MDM2 relationship was investigated and the mechanism leading to upregulation of MDM2, and potentially also aggressiveness of RCC, was proposed based on the results obtained in this study. It was shown here that upregulated p53 is usually wt in RCC and that this p53 drives expression of MDM2 which was subsequently shown to induce cell motility in RCC cell lines. Since increased motility is a common feature of metastatic cells, it can therefore be suggested that p53 acquires oncogenic potential in this particular setting, as it indirectly increases motility and potentially also metastatic expansiveness of these cells.

The ultimate purpose of this study was to identify novel MDM2-binding proteins whose normal function would be altered by high levels of MDM2 resulting in an aggressive phenotype of RCC cells. To accomplish this, the yeast two-hybrid screen of the cDNA library constructed from the RCC cell line overexpressing high levels of p53 and MDM2, was performed. Several novel, putative MDM2 interacting proteins were identified. One of them, NME2, was thoroughly studied. It was demonstrated, that NME2 reduces activity of p53, leads to reduction of the MDM2 levels and influences the MDM2 mediated post-translational modifications of p53 in transfection experiments. Moreover, high levels of MDM2 in RCC cell lines were shown to oppose the NME2-mediated motility suppression, whereas NME2 was able to act as a motility suppressor in the RCC cell lines naturally expressing low levels of MDM2 or cell lines in which the high level of MDM2 was reduced using siRNA. These results suggest a possible mechanism of p53-promoted-MDM2-mediated

action which, by opposing (or directly blocking) the NME2 mediated suppression of motility (thus inducing cell motility), could potentially increase aggressiveness of RCC cells. This issue will be addressed in future experiments using mouse xenograft experiments in order to evaluate significance of this mechanism in tumour progression.

Tumour progression and metastasis is a complex process which is not dependent only on increased motility. Therefore, there is a need for identification of other MDM2 interacting proteins which could be involved in promoting other features of aggressive RCC: resistance to therapies, formation of micro- and macrometastases and could potentially constitute targets for drug design. It is also vital to investigate the reason for which the levels of p53 are elevated in RCC and why do they not trigger apoptosis.

6 References

AGARWAL, M. L., AGARWAL, A., TAYLOR, W. R. & STARK, G. R. (1995) p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A,* 92, 8493-7.

AGOFF, S. N., HOU, J., LINZER, D. I. & WU, B. (1993) Regulation of the human hsp70 promoter by p53. *Science,* 259, 84-7.

ARGENTINI, M., BARBOULE, N. & WASYLYK, B. (2000) The contribution of the RING finger domain of MDM2 to cell cycle progression. *Oncogene,* 19, 3849-57.

ARNAUD-DABERNAT, S., BOURBON, P. M., DIERICH, A., LE MEUR, M. & DANIEL, J. Y. (2003) Knockout mice as model systems for studying nm23/NDP kinase gene functions. Application to the nm23-M1 gene. *J Bioenerg Biomembr,* 35, 19-30.

ASAHARA, H., LI, Y., FUSS, J., HAINES, D. S., VLATKOVIC, N., BOYD, M. T. & LINN, S. (2003) Stimulation of human DNA polymerase epsilon by MDM2. *Nucleic Acids Res,* 31, 2451-9.

BAKER, S. J., FEARON, E. R., NIGRO, J. M., HAMILTON, S. R., PREISINGER, A. C., JESSUP, J. M., VANTUINEN, P., LEDBETTER, D. H., BARKER, D. F., NAKAMURA, Y., WHITE, R. & VOGELSTEIN, B. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science,* 244, 217-21.

BARANOV, P. V., HAMMER, A. W., ZHOU, J., GESTELAND, R. F. & ATKINS, J. F. (2005) Transcriptional slippage in bacteria: distribution in sequenced genomes and utilization in IS element gene expression. *Genome Biol,* 6, R25.

BARBOZA, J. A., IWAKUMA, T., TERZIAN, T., EL-NAGGAR, A. K. & LOZANO, G. (2008) Mdm2 and Mdm4 loss regulates distinct p53 activities. *Mol Cancer Res,* 6, 947-54.

BEISLAND, C. & BEISLAND, H. O. (2004) Natural and clinical course of renal cell carcinoma--better prospect for the patients. *Scand J Surg,* 93, 97-101.

BENNETT, M., MACDONALD, K., CHAN, S. W., LUZIO, J. P., SIMARI, R. & WEISSBERG, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science,* 282, 290-3.

BENSON, K. F., PERSON, R. E., LI, F. Q., WILLIAMS, K. & HORWITZ, M. (2004) Paradoxical homozygous expression from heterozygotes and heterozygous expression from homozygotes as a consequence of transcriptional infidelity through a polyadenine tract in the AP3B1 gene responsible for canine cyclic neutropenia. *Nucleic Acids Res,* 32, 6327-33.

BIRNBOIM, H. C. & DOLY, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res,* 7, 1513-23.

BODE, A. M. & DONG, Z. (2004) Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer,* 4, 793-805.

BOESTEN, L. S., ZADELAAR, S. M., DE CLERCQ, S., FRANCOZ, S., VAN NIEUWKOOP, A., BIESSEN, E. A., HOFMANN, F., FEIL, S., FEIL, R., JOCHEMSEN, A. G., ZURCHER, C., HAVEKES, L. M., VAN VLIJMEN, B. J. & MARINE, J. C. (2006) Mdm2, but not Mdm4, protects terminally

differentiated smooth muscle cells from p53-mediated caspase-3-independent cell death. *Cell Death Differ,* 13, 2089-98.

BOISSAN, M., WENDUM, D., ARNAUD-DABERNAT, S., MUNIER, A., DEBRAY, M., LASCU, I., DANIEL, J. Y. & LACOMBE, M. L. (2005) Increased lung metastasis in transgenic NM23-Null/SV40 mice with hepatocellular carcinoma. *J Natl Cancer Inst,* 97, 836-45.

BOND, G. L., HU, W., BOND, E. E., ROBINS, H., LUTZKER, S. G., ARVA, N. C., BARGONETTI, J., BARTEL, F., TAUBERT, H., WUERL, P., ONEL, K., YIP, L., HWANG, S. J., STRONG, L. C., LOZANO, G. & LEVINE, A. J. (2004) A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell,* 119, 591-602.

BOND, G. L., HU, W. & LEVINE, A. (2005) A single nucleotide polymorphism in the MDM2 gene: from a molecular and cellular explanation to clinical effect. *Cancer Res,* 65, 5481-4.

BOT, F. J., GODSCHALK, J. C., KRISHNADATH, K. K., VAN DER KWAST, T. H. & BOSMAN, F. T. (1994) Prognostic factors in renal-cell carcinoma: immunohistochemical detection of p53 protein versus clinico-pathological parameters. *Int J Cancer,* 57, 634-7.

BOYD, M. T., VLATKOVIC, N. & HAINES, D. S. (2000) A novel cellular protein (MTBP) binds to MDM2 and induces a G1 arrest that is suppressed by MDM2. *J Biol Chem,* 275, 31883-90.

BRAGADO, P., ARMESILLA, A., SILVA, A. & PORRAS, A. (2007) Apoptosis by cisplatin requires p53 mediated p38alpha MAPK activation through ROS generation. *Apoptosis,* 12, 1733-42.

BROOKS, C. L., LI, M. & GU, W. (2004) Monoubiquitination: the signal for p53 nuclear export? *Cell Cycle,* 3, 436-8.

BUESO-RAMOS, C. E., YANG, Y., DELEON, E., MCCOWN, P., STASS, S. A. & ALBITAR, M. (1993) The human MDM-2 oncogene is overexpressed in leukemias. *Blood,* 82, 2617-23.

BUI, M. H., SELIGSON, D., HAN, K. R., PANTUCK, A. J., DOREY, F. J., HUANG, Y., HORVATH, S., LEIBOVICH, B. C., CHOPRA, S., LIAO, S. Y., STANBRIDGE, E., LERMAN, M. I., PALOTIE, A., FIGLIN, R. A. & BELLDEGRUN, A. S. (2003) Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. *Clin Cancer Res,* 9, 802-11.

BUSCHMANN, T., FUCHS, S. Y., LEE, C. G., PAN, Z. Q. & RONAI, Z. (2000) SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell,* 101, 753-62.

CAHILLY-SNYDER, L., YANG-FENG, T., FRANCKE, U. & GEORGE, D. L. (1987) Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat Cell Mol Genet,* 13, 235-44.

CAMPISI, J. (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell,* 120, 513-22.

CAMPLEJOHN, R. S. & RUTHERFORD, J. (2001) p53 functional assays: detecting p53 mutations in both the germline and in sporadic tumours. *Cell Prolif,* 34, 1-14.

CARTER, S., BISCHOF, O., DEJEAN, A. & VOUSDEN, K. H. (2007) C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. *Nat Cell Biol,* 9, 428-35.

CECCONI, F. & GRUSS, P. (2001) Apaf1 in developmental apoptosis and cancer: how many ways to die? *Cell Mol Life Sci,* 58, 1688-97.

CHEMERIS, G., LOKTINOV, A., REMPEL, A., SCHWARZ, M. & BANNASCH, P. (1995) Elevated content of p53 protein in the absence of p53 gene mutations as a possible prognostic marker for human renal cell tumors. *Virchows Arch,* 426, 563-9.

CHEN, D., ZHANG, Z., LI, M., WANG, W., LI, Y., RAYBURN, E. R., HILL, D. L., WANG, H. & ZHANG, R. (2007) Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. *Oncogene,* 26, 5029-37.

CHILOSI, M., DOGLIONI, C., MENESTRINA, F., MONTAGNA, L., RIGO, A., LESTANI, M., BARBARESCHI, M., SCARPA, A., MARIUZZI, G. M. & PIZZOLO, G. (1994) Abnormal expression of the p53-binding protein MDM2 in Hodgkin's disease. *Blood,* 84, 4295-300.

CHIPUK, J. E., BOUCHIER-HAYES, L., KUWANA, T., NEWMEYER, D. D. & GREEN, D. R. (2005) PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science,* 309, 1732-5.

CHO, D. S., JOO, H. J., OH, D. K., KANG, J. H., KIM, Y. S., LEE, K. B. & KIM, S. J. (2005) Cyclooxygenase-2 and p53 expression as prognostic indicators in conventional renal cell carcinoma. *Yonsei Med J,* 46, 133-40.

CHO, Y., GORINA, S., JEFFREY, P. D. & PAVLETICH, N. P. (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science,* 265, 346-55.

CHOI, Y. J., MENDOZA, L., RHA, S. J., SHEIKH-HAMAD, D., BARANOWSKA-DACA, E., NGUYEN, V., SMITH, C. W., NASSAR, G., SUKI, W. N. & TRUONG, L. D. (2001) Role of p53-dependent activation of caspases in chronic obstructive uropathy: evidence from p53 null mutant mice. *J Am Soc Nephrol,* 12, 983-92.

CORDON-CARDO, C., LATRES, E., DROBNJAK, M., OLIVA, M. R., POLLACK, D., WOODRUFF, J. M., MARECHAL, V., CHEN, J., BRENNAN, M. F. & LEVINE, A. J. (1994) Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res,* 54, 794-9.

CRAVEN, R. A. & BANKS, R. E. (2008) Understanding and managing renal cell carcinoma: can proteomic studies contribute to clinical practice? *Contrib Nephrol,* 160, 88-106.

DANOVI, D., MEULMEESTER, E., PASINI, D., MIGLIORINI, D., CAPRA, M., FRENK, R., DE GRAAF, P., FRANCOZ, S., GASPARINI, P., GOBBI, A., HELIN, K., PELICCI, P. G., JOCHEMSEN, A. G. & MARINE, J. C. (2004) Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol,* 24, 5835-43.

DAVIDOFF, A. M., HUMPHREY, P. A., IGLEHART, J. D. & MARKS, J. R. (1991) Genetic basis for p53 overexpression in human breast cancer. *Proc Natl Acad Sci U S A,* 88, 5006-10.

DE LA ROSA, A., WILLIAMS, R. L. & STEEG, P. S. (1995) Nm23/nucleoside diphosphate kinase: toward a structural and biochemical understanding of its biological functions. *Bioessays,* 17, 53-62.

DELAHUNT, B. (1999) Sarcomatoid renal carcinoma: the final common dedifferentiation pathway of renal epithelial malignancies. *Pathology,* 31, 185-90.

DELEO, A. B., JAY, G., APPELLA, E., DUBOIS, G. C., LAW, L. W. & OLD, L. J. (1979) Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A,* 76, 2420-4.

DIPPOLD, W. G., JAY, G., DELEO, A. B., KHOURY, G. & OLD, L. J. (1981) p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. *Proc Natl Acad Sci U S A,* 78, 1695-9.

DONEHOWER, L. A., HARVEY, M., SLAGLE, B. L., MCARTHUR, M. J., MONTGOMERY, C. A., JR., BUTEL, J. S. & BRADLEY, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature,* 356, 215-21.

DUAN, D. R., PAUSE, A., BURGESS, W. H., ASO, T., CHEN, D. Y., GARRETT, K. P., CONAWAY, R. C., CONAWAY, J. W., LINEHAN, W. M. & KLAUSNER, R. D. (1995) Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science,* 269, 1402-6.

EISCHEN, C. M., WEBER, J. D., ROUSSEL, M. F., SHERR, C. J. & CLEVELAND, J. L. (1999) Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev,* 13, 2658-69.

EL-DEIRY, W. S., KERN, S. E., PIETENPOL, J. A., KINZLER, K. W. & VOGELSTEIN, B. (1992) Definition of a consensus binding site for p53. *Nat Genet,* 1, 45-9.

ELENBAAS, B., DOBBELSTEIN, M., ROTH, J., SHENK, T. & LEVINE, A. J. (1996) The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol Med,* 2, 439-51.

ELIYAHU, D., GOLDFINGER, N., PINHASI-KIMHI, O., SHAULSKY, G., SKURNIK, Y., ARAI, N., ROTTER, V. & OREN, M. (1988) Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene,* 3, 313-21.

ELMORE, J. M., KADESKY, K. T., KOENEMAN, K. S. & SAGALOWSKY, A. I. (2003) Reassessment of the 1997 TNM classification system for renal cell carcinoma. *Cancer,* 98, 2329-34.

ELMORE, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol,* 35, 495-516.

FAKHARZADEH, S. S., TRUSKO, S. P. & GEORGE, D. L. (1991) Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *Embo J,* 10, 1565-9.

FANG, S., JENSEN, J. P., LUDWIG, R. L., VOUSDEN, K. H. & WEISSMAN, A. M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem,* 275, 8945-51.

FARABAUGH, P. J. & BJORK, G. R. (1999) How translational accuracy influences reading frame maintenance. *Embo J,* 18, 1427-34.

FDA U.S. Food and Drug Adnimistration, http://www.fda.gov/.

FICARRA, V., GALFANO, A., MANCINI, M., MARTIGNONI, G. & ARTIBANI, W. (2007) TNM staging system for renal-cell carcinoma: current status and future perspectives. *Lancet Oncol,* 8, 554-8.

FIELDS, S. & SONG, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature,* 340, 245-6.

FINCH, R. A., DONOVIEL, D. B., POTTER, D., SHI, M., FAN, A., FREED, D. D., WANG, C. Y., ZAMBROWICZ, B. P., RAMIREZ-SOLIS, R., SANDS, A. T. & ZHANG, N. (2002) mdmx is a negative regulator of p53 activity in vivo. *Cancer Res,* 62, 3221-5.

FONTOURA, B. M., ATIENZA, C. A., SOROKINA, E. A., MORIMOTO, T. & CARROLL, R. B. (1997) Cytoplasmic p53 polypeptide is associated with ribosomes. *Mol Cell Biol,* 17, 3146-54.

FRANCOZ, S., FROMENT, P., BOGAERTS, S., DE CLERCQ, S., MAETENS, M., DOUMONT, G., BELLEFROID, E. & MARINE, J. C. (2006) Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. *Proc Natl Acad Sci U S A,* 103, 3232-7.

FREEDMAN, D. A., WU, L. & LEVINE, A. J. (1999) Functions of the MDM2 oncoprotein. *Cell Mol Life Sci,* 55, 96-107.

FRIDMAN, J. S., HERNANDO, E., HEMANN, M. T., DE STANCHINA, E., CORDON-CARDO, C. & LOWE, S. W. (2003) Tumor promotion by Mdm2 splice variants unable to bind p53. *Cancer Res,* 63, 5703-6.

FRIEDBERG, E. C. (2001) How nucleotide excision repair protects against cancer. *Nat Rev Cancer,* 1, 22-33.

FUHRMAN, S. A., LASKY, L. C. & LIMAS, C. (1982) Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol,* 6, 655-63.

GAD, S., LEFEVRE, S. H., KHOO, S. K., GIRAUD, S., VIEILLEFOND, A., VASILIU, V., FERLICOT, S., MOLINIE, V., DENOUX, Y., THIOUNN, N., CHRETIEN, Y., MEJEAN, A., ZERBIB, M., BENOIT, G., HERVE, J. M., ALLEGRE, G., BRESSAC-DE PAILLERETS, B., TEH, B. T. & RICHARD, S. (2007) Mutations in BHD and TP53 genes, but not in HNF1beta gene, in a large series of sporadic chromophobe renal cell carcinoma. *Br J Cancer,* 96, 336-40.

GANGULI, G. & WASYLYK, B. (2003) p53-independent functions of MDM2. *Mol Cancer Res,* 1, 1027-35.

GAO, C., ZOU, Z., XU, L., MOUL, J., SETH, P. & SRIVASTAVA, S. (2000) p53-dependent induction of heat shock protein 27 (HSP27) expression. *Int J Cancer,* 88, 191-4.

GELB, A. B., SUDILOVSKY, D., WU, C. D., WEISS, L. M. & MEDEIROS, L. J. (1997) Appraisal of intratumoral microvessel density, MIB-1 score, DNA content, and p53 protein expression as prognostic indicators in patients with locally confined renal cell carcinoma. *Cancer,* 80, 1768-75.

GIRGIN, C., TARHAN, H., HEKIMGIL, M., SEZER, A. & GUREL, G. (2001) P53 mutations and other prognostic factors of renal cell carcinoma. *Urol Int,* 66, 78-83.

GNARRA, J. R., TORY, K., WENG, Y., SCHMIDT, L., WEI, M. H., LI, H., LATIF, F., LIU, S., CHEN, F., DUH, F. M. & ET AL. (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet,* 7, 85-90.

GRIER, J. D., XIONG, S., ELIZONDO-FRAIRE, A. C., PARANT, J. M. & LOZANO, G. (2006) Tissue-specific differences of p53 inhibition by Mdm2 and Mdm4. *Mol Cell Biol,* 26, 192-8.

GU, J., KAWAI, H., NIE, L., KITAO, H., WIEDERSCHAIN, D., JOCHEMSEN, A. G., PARANT, J., LOZANO, G. & YUAN, Z. M. (2002) Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *J Biol Chem,* 277, 19251-4.

GUROVA, K. V., HILL, J. E., RAZORENOVA, O. V., CHUMAKOV, P. M. & GUDKOV, A. V. (2004) p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. *Cancer Res,* 64, 1951-8.

HAINAUT, P. & MANN, K. (2001) Zinc binding and redox control of p53 structure and function. *Antioxid Redox Signal,* 3, 611-23.

HAITEL, A., WIENER, H. G., BAETHGE, U., MARBERGER, M. & SUSANI, M. (2000) mdm2 expression as a prognostic indicator in clear cell renal cell carcinoma: comparison with p53 overexpression and clinicopathological parameters. *Clin Cancer Res,* 6, 1840-4.

HALAZONETIS, T. D., GORGOULIS, V. G. & BARTEK, J. (2008) An oncogene-induced DNA damage model for cancer development. *Science,* 319, 1352-5.

HAMMOND, E. M., DORIE, M. J. & GIACCIA, A. J. (2003) ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem,* 278, 12207-13.

HAMMOND, E. M., KAUFMANN, M. R. & GIACCIA, A. J. (2007) Oxygen sensing and the DNA-damage response. *Curr Opin Cell Biol,* 19, 680-4.

HARVEY, M., MCARTHUR, M. J., MONTGOMERY, C. A., JR., BUTEL, J. S., BRADLEY, A. & DONEHOWER, L. A. (1993) Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat Genet,* 5, 225-9.

HEMANN, M. T. & NARITA, M. (2007) Oncogenes and senescence: breaking down in the fast lane. *Genes Dev,* 21, 1-5.

HINDS, P., FINLAY, C. & LEVINE, A. J. (1989) Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. *J Virol,* 63, 739-46.

HINDS, P. W., FINLAY, C. A., FREY, A. B. & LEVINE, A. J. (1987) Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. *Mol Cell Biol,* 7, 2863-9.

HIRATA, H., HINODA, Y., KIKUNO, N., KAWAMOTO, K., SUEHIRO, Y., TANAKA, Y. & DAHIYA, R. (2007) MDM2 SNP309 polymorphism as risk factor for susceptibility and poor prognosis in renal cell carcinoma. *Clin Cancer Res,* 13, 4123-9.

HOFMOCKEL, G., WITTMANN, A., DAMMRICH, J. & BASSUKAS, I. D. (1996) Expression of p53 and bcl-2 in primary locally confined renal cell carcinomas: no evidence for prognostic significance. *Anticancer Res,* 16, 3807-11.

HOLLANDER, M. C., SHEIKH, M. S., BULAVIN, D. V., LUNDGREN, K., AUGERI-HENMUELLER, L., SHEHEE, R., MOLINARO, T. A., KIM, K. E., TOLOSA, E., ASHWELL, J. D., ROSENBERG, M. P., ZHAN, Q., FERNANDEZ-SALGUERO, P. M., MORGAN, W. F., DENG, C. X. & FORNACE, A. J., JR. (1999) Genomic instability in Gadd45a-deficient mice. *Nat Genet,* 23, 176-84.

HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B. & HARRIS, C. C. (1991) p53 mutations in human cancers. *Science,* 253, 49-53.

HONDA, R., TANAKA, H. & YASUDA, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett,* 420, 25-7.

HONDA, R. & YASUDA, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J,* 18, 22-7.

HONDA, R. & YASUDA, H. (2000) Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. *Oncogene,* 19, 1473-6.

HORAK, C. E., LEE, J. H., ELKAHLOUN, A. G., BOISSAN, M., DUMONT, S., MAGA, T. K., ARNAUD-DABERNAT, S., PALMIERI, D., STETLER-STEVENSON, W. G., LACOMBE, M. L., MELTZER, P. S. & STEEG, P. S. (2007a) Nm23-H1 suppresses tumor cell motility by down-regulating the lysophosphatidic acid receptor EDG2. *Cancer Res,* 67, 7238-46.

HORAK, C. E., MENDOZA, A., VEGA-VALLE, E., ALBAUGH, M., GRAFF-CHERRY, C., MCDERMOTT, W. G., HUA, E., MERINO, M. J., STEINBERG, S. M., KHANNA, C. & STEEG, P. S. (2007b) Nm23-H1 suppresses metastasis by inhibiting expression of the lysophosphatidic acid receptor EDG2. *Cancer Res,* 67, 11751-9.

ILIOPOULOS, O. (2006) Molecular biology of renal cell cancer and the identification of therapeutic targets. *J Clin Oncol,* 24, 5593-600.

ILIOPOULOS, O., KIBEL, A., GRAY, S. & KAELIN, W. G., JR. (1995) Tumour suppression by the human von Hippel-Lindau gene product. *Nat Med,* 1, 822-6.

ILIOPOULOS, O., OHH, M. & KAELIN, W. G., JR. (1998) pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A,* 95, 11661-6.

ISOBE, K. (2003) [Stress and aging]. *Nippon Ronen Igakkai Zasshi,* 40, 565-8.

ITAHANA, K., MAO, H., JIN, A., ITAHANA, Y., CLEGG, H. V., LINDSTROM, M. S., BHAT, K. P., GODFREY, V. L., EVAN, G. I. & ZHANG, Y. (2007) Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. *Cancer Cell,* 12, 355-66.

IWAKUMA, T. & LOZANO, G. (2003) MDM2, an introduction. *Mol Cancer Res,* 1, 993-1000.

JACKSON, M. W. & BERBERICH, S. J. (2000) MdmX protects p53 from Mdm2-mediated degradation. *Mol Cell Biol,* 20, 1001-7.

JAYSON, M. & SANDERS, H. (1998) Increased incidence of serendipitously discovered renal cell carcinoma. *Urology,* 51, 203-5.

JOERGER, A. C. & FERSHT, A. R. (2008) Structural biology of the tumor suppressor p53. *Annu Rev Biochem,* 77, 557-82.

JONES, S. N., HANCOCK, A. R., VOGEL, H., DONEHOWER, L. A. & BRADLEY, A. (1998) Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. *Proc Natl Acad Sci U S A,* 95, 15608-12.

JONES, S. N., ROE, A. E., DONEHOWER, L. A. & BRADLEY, A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature,* 378, 206-8.

JORDAN, J. J., MENENDEZ, D., INGA, A., NOURREDINE, M., BELL, D. & RESNICK, M. A. (2008) Noncanonical DNA motifs as transactivation targets by wild type and mutant p53. *PLoS Genet,* 4, e1000104.

JOSEPH, T. W., ZAIKA, A. & MOLL, U. M. (2003) Nuclear and cytoplasmic degradation of endogenous p53 and HDM2 occurs during down-regulation of the p53 response after multiple types of DNA damage. *Faseb J,* 17, 1622-30.

JUNG, H., SEONG, H. A. & HA, H. (2007) NM23-H1 tumor suppressor and its interacting partner STRAP activate p53 function. *J Biol Chem,* 282, 35293-307.

JUNG, S., PAEK, Y. W., MOON, K. S., WEE, S. C., RYU, H. H., JEONG, Y. I., SUN, H. S., JIN, Y. H., KIM, K. K. & AHN, K. Y. (2006) Expression of Nm23 in gliomas and its effect on migration and invasion in vitro. *Anticancer Res,* 26, 249-58.

KAMEL, D., TURPEENNIEMI-HUJANEN, T., VAHAKANGAS, K., PAAKKO, P. & SOINI, Y. (1994) Proliferating cell nuclear antigen but not p53 or human papillomavirus DNA correlates with advanced clinical stage in renal cell carcinoma. *Histopathology,* 25, 339-47.

KAMIJO, T., BODNER, S., VAN DE KAMP, E., RANDLE, D. H. & SHERR, C. J. (1999) Tumor spectrum in ARF-deficient mice. *Cancer Res,* 59, 2217-22.

KANKURI, M., SODERSTROM, K. O., PELLINIEMI, T. T., VAHLBERG, T., PYRHONEN, S. & SALMINEN, E. (2006) The association of immunoreactive p53 and Ki-67 with T-stage, grade, occurrence of metastases and survival in renal cell carcinoma. *Anticancer Res,* 26, 3825-33.

KELLY, K. J., PLOTKIN, Z., VULGAMOTT, S. L. & DAGHER, P. C. (2003) P53 mediates the apoptotic response to GTP depletion after renal ischemia-reperfusion: protective role of a p53 inhibitor. *J Am Soc Nephrol,* 14, 128-38.

KIM, H. L., SELIGSON, D., LIU, X., JANZEN, N., BUI, M. H., YU, H., SHI, T., FIGLIN, R. A., HORVATH, S. & BELLDEGRUN, A. S. (2004) Using protein expressions to predict survival in clear cell renal carcinoma. *Clin Cancer Res,* 10, 5464-71.

KIM, W. Y. & KAELIN, W. G. (2004) Role of VHL gene mutation in human cancer. *J Clin Oncol,* 22, 4991-5004.

KLATTE, T., SELIGSON, D. B., LAROCHELLE, J., SHUCH, B., SAID, J. W., RIGGS, S. B., ZOMORODIAN, N., KABBINAVAR, F. F., PANTUCK, A. J. & BELLDEGRUN, A. S. (2009) Molecular signatures of localized clear cell renal cell carcinoma to predict disease-free survival after nephrectomy. *Cancer Epidemiol Biomarkers Prev,* 18, 894-900.

KLEYMENOVA, E., EVERITT, J. I., PLUTA, L., PORTIS, M., GNARRA, J. R. & WALKER, C. L. (2004) Susceptibility to vascular neoplasms but no increased susceptibility to renal carcinogenesis in Vhl knockout mice. *Carcinogenesis,* 25, 309-15.

KRAMER, B. A., GAO, X., DAVIS, M., HALL, M., HOLZBEIERLEIN, J. & TAWFIK, O. (2005) Prognostic significance of ploidy, MIB-1 proliferation marker, and p53 in renal cell carcinoma. *J Am Coll Surg,* 201, 565-70.

KURZ, E. U. & LEES-MILLER, S. P. (2004) DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst),* 3, 889-900.

LABAER, J., GARRETT, M. D., STEVENSON, L. F., SLINGERLAND, J. M., SANDHU, C., CHOU, H. S., FATTAEY, A. & HARLOW, E. (1997) New functional activities for the p21 family of CDK inhibitors. *Genes Dev,* 11, 847-62.

LACOMBE, M. L., MILON, L., MUNIER, A., MEHUS, J. G. & LAMBETH, D. O. (2000) The human Nm23/nucleoside diphosphate kinases. *J Bioenerg Biomembr,* 32, 247-58.

LAKIN, N. D. & JACKSON, S. P. (1999) Regulation of p53 in response to DNA damage. *Oncogene,* 18, 7644-55.

LAMB, G. W., MCMILLAN, D. C., RAMSEY, S. & AITCHISON, M. (2006) The relationship between the preoperative systemic inflammatory response and cancer-specific survival in patients undergoing potentially curative resection for renal clear cell cancer. *Br J Cancer,* 94, 781-4.

LANE, D. P. & CRAWFORD, L. V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature,* 278, 261-3.

LANGNER, C., RATSCHEK, M., REHAK, P., TSYBROVSKYY, O. & ZIGEUNER, R. (2005) The pT1a and pT1b category subdivision in renal cell carcinoma: is it reflected by differences in tumour biology? *BJU Int,* 95, 310-4.

LEACH, F. S., TOKINO, T., MELTZER, P., BURRELL, M., OLINER, J. D., SMITH, S., HILL, D. E., SIDRANSKY, D., KINZLER, K. W. & VOGELSTEIN, B. (1993) p53 Mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res,* 53, 2231-4.

LEVINE, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell,* 88, 323-31.

LI, K. L., DU, X. L., SONG, R. H., ZHAO, L., HE, Y. N., ZHANG, J. G. & CHEN, L. (2007) [Effect of p53 on the variation of renal tubular epithelial cells after kidney ischemia/reperfusion injury]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi,* 23, 6-10.

LI, M., BROOKS, C. L., WU-BAER, F., CHEN, D., BAER, R. & GU, W. (2003) Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science,* 302, 1972-5.

LINARES, L. K., HENGSTERMANN, A., CIECHANOVER, A., MULLER, S. & SCHEFFNER, M. (2003) HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *Proc Natl Acad Sci U S A,* 100, 12009-14.

LINEHAN, W. M., LERMAN, M. I. & ZBAR, B. (1995) Identification of the von Hippel-Lindau (VHL) gene. Its role in renal cancer. *Jama,* 273, 564-70.

LINTON, M. F., RAABE, M., PIEROTTI, V. & YOUNG, S. G. (1997) Reading-frame restoration by transcriptional slippage at long stretches of adenine residues in mammalian cells. *J Biol Chem,* 272, 14127-32.

LINZER, D. I. & LEVINE, A. J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell,* 17, 43-52.

LIPPONEN, P., ESKELINEN, M., HIETALA, K., SYRJANEN, K. & GAMBETTA, R. A. (1994) Expression of proliferating cell nuclear antigen (PC10), p53 protein and c-erbB-2 in renal adenocarcinoma. *Int J Cancer,* 57, 275-80.

LITTLE, N. A. & JOCHEMSEN, A. G. (2001) Hdmx and Mdm2 can repress transcription activation by p53 but not by p63. *Oncogene,* 20, 4576-80.

LIU, B., CHEN, Y. & ST CLAIR, D. K. (2008) ROS and p53: a versatile partnership. *Free Radic Biol Med,* 44, 1529-35.

LJUNGBERG, B., BOZOKY, B., KOVACS, G., STATTIN, P., FARRELLY, E., NYLANDER, K. & LANDBERG, G. (2001) p53 expression in correlation to clinical outcome in patients with renal cell carcinoma. *Scand J Urol Nephrol,* 35, 15-20.

LOPEZ-BELTRAN, A., CARRASCO, J. C., CHENG, L., SCARPELLI, M., KIRKALI, Z. & MONTIRONI, R. (2009) 2009 update on the classification of renal epithelial tumors in adults. *Int J Urol,* 16, 432-43.

LOWE, S. W., CEPERO, E. & EVAN, G. (2004) Intrinsic tumour suppression. *Nature,* 432, 307-15.

LOZANO, G. & ZAMBETTI, G. P. (2005) What have animal models taught us about the p53 pathway? *J Pathol,* 205, 206-20.

LU, M. L., WIKMAN, F., ORNTOFT, T. F., CHARYTONOWICZ, E., RABBANI, F., ZHANG, Z., DALBAGNI, G., POHAR, K. S., YU, G. & CORDON-CARDO, C. (2002) Impact of alterations affecting the p53 pathway in bladder cancer on clinical outcome, assessed by conventional and array-based methods. *Clin Cancer Res,* 8, 171-9.

LUNDGREN, K., MONTES DE OCA LUNA, R., MCNEILL, Y. B., EMERICK, E. P., SPENCER, B., BARFIELD, C. R., LOZANO, G., ROSENBERG, M. P. & FINLAY, C. A. (1997) Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes Dev,* 11, 714-25.

MACCALLUM, D. E., HUPP, T. R., MIDGLEY, C. A., STUART, D., CAMPBELL, S. J., HARPER, A., WALSH, F. S., WRIGHT, E. G., BALMAIN, A., LANE, D. P. & HALL, P. A. (1996) The p53 response to

ionising radiation in adult and developing murine tissues. *Oncogene,* 13, 2575-87.

MAESTRO, R., GLOGHINI, A., DOGLIONI, C., GASPAROTTO, D., VUKOSAVLJEVIC, T., DE RE, V., LAURINO, L., CARBONE, A. & BOIOCCHI, M. (1995) MDM2 overexpression does not account for stabilization of wild-type p53 protein in non-Hodgkin's lymphomas. *Blood,* 85, 3239-46.

MAGUIRE, M., NIELD, P. C., DEVLING, T., JENKINS, R. E., PARK, B. K., POLANSKI, R., VLATKOVIC, N. & BOYD, M. T. (2008) MDM2 regulates dihydrofolate reductase activity through monoubiquitination. *Cancer Res,* 68, 3232-42.

MAHER, E. R., YATES, J. R., HARRIES, R., BENJAMIN, C., HARRIS, R., MOORE, A. T. & FERGUSON-SMITH, M. A. (1990) Clinical features and natural history of von Hippel-Lindau disease. *Q J Med,* 77, 1151-63.

MARINE, J. C., FRANCOZ, S., MAETENS, M., WAHL, G., TOLEDO, F. & LOZANO, G. (2006) Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. *Cell Death Differ,* 13, 927-34.

MARINE, J. C. & JOCHEMSEN, A. G. (2005) Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun,* 331, 750-60.

MARKS, J. R., DAVIDOFF, A. M., KERNS, B. J., HUMPHREY, P. A., PENCE, J. C., DODGE, R. K., CLARKE-PEARSON, D. L., IGLEHART, J. D., BAST, R. C., JR. & BERCHUCK, A. (1991) Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res,* 51, 2979-84.

MARTIN, K., TROUCHE, D., HAGEMEIER, C., SORENSEN, T. S., LA THANGUE, N. B. & KOUZARIDES, T. (1995) Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature,* 375, 691-4.

MAYA, R., BALASS, M., KIM, S. T., SHKEDY, D., LEAL, J. F., SHIFMAN, O., MOAS, M., BUSCHMANN, T., RONAI, Z., SHILOH, Y., KASTAN, M. B., KATZIR, E. & OREN, M. (2001) ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev,* 15, 1067-77.

MAYO, L. D. & DONNER, D. B. (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A,* 98, 11598-603.

MAYO, L. D. & DONNER, D. B. (2002) The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci,* 27, 462-7.

MAYO, L. D., TURCHI, J. J. & BERBERICH, S. J. (1997) Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res,* 57, 5013-6.

MEEK, D. W. & KNIPPSCHILD, U. (2003) Posttranslational modification of MDM2. *Mol Cancer Res,* 1, 1017-26.

MELCHIOR, F. & HENGST, L. (2002) SUMO-1 and p53. *Cell Cycle,* 1, 245-9.

MENDRYSA, S. M., MCELWEE, M. K., MICHALOWSKI, J., O'LEARY, K. A., YOUNG, K. M. & PERRY, M. E. (2003) mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. *Mol Cell Biol,* 23, 462-72.

MIGLIORINI, D., LAZZERINI DENCHI, E., DANOVI, D., JOCHEMSEN, A., CAPILLO, M., GOBBI, A., HELIN, K., PELICCI, P. G. & MARINE, J. C.

(2002) Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol,* 22, 5527-38.

MIHARA, M., ERSTER, S., ZAIKA, A., PETRENKO, O., CHITTENDEN, T., PANCOSKA, P. & MOLL, U. M. (2003) p53 has a direct apoptogenic role at the mitochondria. *Mol Cell,* 11, 577-90.

MIYAKODA, M., SUZUKI, K., KODAMA, S. & WATANABE, M. (2002) Activation of ATM and phosphorylation of p53 by heat shock. *Oncogene,* 21, 1090-6.

MIYAZAKI, H., FUKUDA, M., ISHIJIMA, Y., TAKAGI, Y., IIMURA, T., NEGISHI, A., HIRAYAMA, R., ISHIKAWA, N., AMAGASA, T. & KIMURA, N. (1999) Overexpression of nm23-H2/NDP kinase B in a human oral squamous cell carcinoma cell line results in reduced metastasis, differentiated phenotype in the metastatic site, and growth factor-independent proliferative activity in culture. *Clin Cancer Res,* 5, 4301-7.

MOCH, H., SAUTER, G., GASSER, T. C., BUCHHOLZ, N., BUBENDORF, L., RICHTER, J., JIANG, F., DELLAS, A. & MIHATSCH, M. J. (1997) p53 protein expression but not mdm-2 protein expression is associated with rapid tumor cell proliferation and prognosis in renal cell carcinoma. *Urol Res,* 25 Suppl 1, S25-30.

MOMAND, J., ZAMBETTI, G. P., OLSON, D. C., GEORGE, D. & LEVINE, A. J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell,* 69, 1237-45.

MONTES DE OCA LUNA, R., WAGNER, D. S. & LOZANO, G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature,* 378, 203-6.

MOOI, W. J. & PEEPER, D. S. (2006) Oncogene-induced cell senescence--halting on the road to cancer. *N Engl J Med,* 355, 1037-46.

MOTTAGUI-TABAR, S., TUITE, M. F. & ISAKSSON, L. A. (1998) The influence of 5' codon context on translation termination in Saccharomyces cerevisiae. *Eur J Biochem,* 257, 249-54.

MOTZER, R. J., BANDER, N. H. & NANUS, D. M. (1996) Renal-cell carcinoma. *N Engl J Med,* 335, 865-75.

MOTZER, R. J., MICHAELSON, M. D., REDMAN, B. G., HUDES, G. R., WILDING, G., FIGLIN, R. A., GINSBERG, M. S., KIM, S. T., BAUM, C. M., DEPRIMO, S. E., LI, J. Z., BELLO, C. L., THEUER, C. P., GEORGE, D. J. & RINI, B. I. (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol,* 24, 16-24.

MUNRO, A. J., LAIN, S. & LANE, D. P. (2005) P53 abnormalities and outcomes in colorectal cancer: a systematic review. *Br J Cancer,* 92, 434-44.

NAMY, O., HATIN, I. & ROUSSET, J. P. (2001) Impact of the six nucleotides downstream of the stop codon on translation termination. *EMBO Rep,* 2, 787-93.

NICE, National Institute for Health and Clinical Excellence Technology Appraisal guidance 178, http://www.nice.org.uk/nicemedia/live/12220/45232/45232.pdf

NICE, National Institute for Health and Clinical Excellence, Guidance on Cancer Services, http://www.nice.org.uk/nicemedia/pdf/Urological_Manual.pdf

NOON, A. P., VLATKOVIC, N., POLANSKI, R., MAGUIRE, M., SHAWKI, H., PARSONS, K. & BOYD, M. T. p53 and MDM2 in renal cell carcinoma: biomarkers for disease progression and future therapeutic targets? *Cancer.*

O'CONNOR, M. (1998) tRNA imbalance promotes -1 frameshifting via near-cognate decoding. *J Mol Biol,* 279, 727-36.

ODA, H., NAKATSURU, Y. & ISHIKAWA, T. (1995) Mutations of the p53 gene and p53 protein overexpression are associated with sarcomatoid transformation in renal cell carcinomas. *Cancer Res,* 55, 658-62.

OFIR-ROSENFELD, Y., BOGGS, K., MICHAEL, D., KASTAN, M. B. & OREN, M. (2008) Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol Cell,* 32, 180-9.

OGAWARA, Y., KISHISHITA, S., OBATA, T., ISAZAWA, Y., SUZUKI, T., TANAKA, K., MASUYAMA, N. & GOTOH, Y. (2002) Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J Biol Chem,* 277, 21843-50.

OHH, M. (2006) Ubiquitin pathway in VHL cancer syndrome. *Neoplasia,* 8, 623-9.

OHMIYA, N., TAGUCHI, A., MABUCHI, N., ITOH, A., HIROOKA, Y., NIWA, Y. & GOTO, H. (2006) MDM2 promoter polymorphism is associated with both an increased susceptibility to gastric carcinoma and poor prognosis. *J Clin Oncol,* 24, 4434-40.

OLINER, J. D., KINZLER, K. W., MELTZER, P. S., GEORGE, D. L. & VOGELSTEIN, B. (1992) Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature,* 358, 80-3.

OLINER, J. D., PIETENPOL, J. A., THIAGALINGAM, S., GYURIS, J., KINZLER, K. W. & VOGELSTEIN, B. (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature,* 362, 857-60.

OLUMI, A. F., WEIDNER, N. & PRESTI, J. C. (2001) p53 immunoreactivity correlates with Ki-67 and bcl-2 expression in renal cell carcinoma. *Urol Oncol,* 6, 63-67.

OTERO, A. S. (2000) NM23/nucleoside diphosphate kinase and signal transduction. *J Bioenerg Biomembr,* 32, 269-75.

OTSUKI, Y., TANAKA, M., YOSHII, S., KAWAZOE, N., NAKAYA, K. & SUGIMURA, H. (2001) Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. *Proc Natl Acad Sci U S A,* 98, 4385-90.

PADHANI, A. R. & OLLIVIER, L. (2001) The RECIST (Response Evaluation Criteria in Solid Tumors) criteria: implications for diagnostic radiologists. *Br J Radiol,* 74, 983-6.

PAPADOPOULOS, I., RUDOLPH, P. & WEICHERT-JACOBSEN, K. (1997) Value of p53 expression, cellular proliferation, and DNA content as prognostic indicators in renal cell carcinoma. *Eur Urol,* 32, 110-7.

PARANT, J., CHAVEZ-REYES, A., LITTLE, N. A., YAN, W., REINKE, V., JOCHEMSEN, A. G. & LOZANO, G. (2001) Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat Genet,* 29, 92-5.

PARK, S. H., PARK, J. Y. & WEISS, R. H. (2008) Antisense attenuation of p21 sensitizes kidney cancer to apoptosis in response to conventional DNA

damaging chemotherapy associated with enhancement of phospho-p53. *J Urol,* 180, 352-60.

PATEL, P. H., CHAGANTI, R. S. & MOTZER, R. J. (2006) Targeted therapy for metastatic renal cell carcinoma. *Br J Cancer,* 94, 614-9.

PERRET, A. G., CLEMENCON, A., LI, G., TOSTAIN, J. & PEOC'H, M. (2008) Differential expression of prognostic markers in histological subtypes of papillary renal cell carcinoma. *BJU Int,* 102, 183-7.

PERSONS, D. L., YAZLOVITSKAYA, E. M. & PELLING, J. C. (2000) Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. *J Biol Chem,* 275, 35778-85.

PHUOC, N. B., EHARA, H., GOTOH, T., NAKANO, M., YOKOI, S., DEGUCHI, T. & HIROSE, Y. (2007) Immunohistochemical analysis with multiple antibodies in search of prognostic markers for clear cell renal cell carcinoma. *Urology,* 69, 843-8.

POLLOCK, P. M., PEARSON, J. V. & HAYWARD, N. K. (1996) Compilation of somatic mutations of the CDKN2 gene in human cancers: non-random distribution of base substitutions. *Genes Chromosomes Cancer,* 15, 77-88.

POMERANTZ, J., SCHREIBER-AGUS, N., LIEGEOIS, N. J., SILVERMAN, A., ALLAND, L., CHIN, L., POTES, J., CHEN, K., ORLOW, I., LEE, H. W., CORDON-CARDO, C. & DEPINHO, R. A. (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell,* 92, 713-23.

POSTEL, E. H., ABRAMCZYK, B. A., GURSKY, S. K. & XU, Y. (2002) Structure-based mutational and functional analysis identify human NM23-H2 as a multifunctional enzyme. *Biochemistry,* 41, 6330-7.

POSTEL, E. H., BERBERICH, S. J., FLINT, S. J. & FERRONE, C. A. (1993) Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science,* 261, 478-80.

POSTEL, E. H., WEISS, V. H., BENEKEN, J. & KIRTANE, A. (1996) Mutational analysis of NM23-H2/NDP kinase identifies the structural domains critical to recognition of a c-myc regulatory element. *Proc Natl Acad Sci U S A,* 93, 6892-7.

PRIVES, C. (1998) Signaling to p53: breaking the MDM2-p53 circuit. *Cell,* 95, 5-8.

RATINIER, M., BOULANT, S., COMBET, C., TARGETT-ADAMS, P., MCLAUCHLAN, J. & LAVERGNE, J. P. (2008) Transcriptional slippage prompts recoding in alternate reading frames in the hepatitis C virus (HCV) core sequence from strain HCV-1. *J Gen Virol,* 89, 1569-78.

RAVI, R., MOOKERJEE, B., BHUJWALLA, Z. M., SUTTER, C. H., ARTEMOV, D., ZENG, Q., DILLEHAY, L. E., MADAN, A., SEMENZA, G. L. & BEDI, A. (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev,* 14, 34-44.

RAYNER, K., CHEN, Y. X., HIBBERT, B., WHITE, D., MILLER, H., POSTEL, E. H. & O'BRIEN, E. R. (2008) Discovery of NM23-H2 as an estrogen receptor beta-associated protein: role in estrogen-induced gene transcription and cell migration. *J Steroid Biochem Mol Biol,* 108, 72-81.

REEVES, D. J. & LIU, C. Y. (2009) Treatment of metastatic renal cell carcinoma. *Cancer Chemother Pharmacol,* 64, 11-25.

RIED, T., JUST, K. E., HOLTGREVE-GREZ, H., DU MANOIR, S., SPEICHER, M. R., SCHROCK, E., LATHAM, C., BLEGEN, H., ZETTERBERG, A.,

CREMER, T. & ET AL. (1995) Comparative genomic hybridization of formalin-fixed, paraffin-embedded breast tumors reveals different patterns of chromosomal gains and losses in fibroadenomas and diploid and aneuploid carcinomas. *Cancer Res,* 55, 5415-23.

RIEMENSCHNEIDER, M. J., KNOBBE, C. B. & REIFENBERGER, G. (2003) Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int J Cancer,* 104, 752-7.

RINI, B. I. (2008) Temsirolimus, an inhibitor of mammalian target of rapamycin. *Clin Cancer Res,* 14, 1286-90.

RINKER-SCHAEFFER, C. W., CHEKMAREVA, M. A. & MOHLER, J. L. (1996) The role of motility proteins and metastasis-suppressor genes in prostate cancer progression. *Stem Cells,* 14, 508-16.

RIOUX-LECLERCQ, N., TURLIN, B., BANSARD, J., PATARD, J., MANUNTA, A., MOULINOUX, J. P., GUILLE, F., RAMEE, M. P. & LOBEL, B. (2000) Value of immunohistochemical Ki-67 and p53 determinations as predictive factors of outcome in renal cell carcinoma. *Urology,* 55, 501-5.

ROE, J. S., KIM, H., LEE, S. M., KIM, S. T., CHO, E. J. & YOUN, H. D. (2006) p53 stabilization and transactivation by a von Hippel-Lindau protein. *Mol Cell,* 22, 395-405.

ROUVIERE, O., BOUVIER, R., NEGRIER, S., BADET, L. & LYONNET, D. (2006) Nonmetastatic renal-cell carcinoma: is it really possible to define rational guidelines for post-treatment follow-up? *Nat Clin Pract Oncol,* 3, 200-13.

RUIJS, M. W., SCHMIDT, M. K., NEVANLINNA, H., TOMMISKA, J., AITTOMAKI, K., PRUNTEL, R., VERHOEF, S. & VAN'T VEER, L. J.

(2007) The single-nucleotide polymorphism 309 in the MDM2 gene contributes to the Li-Fraumeni syndrome and related phenotypes. *Eur J Hum Genet,* 15, 110-4.

SABBATINI, P. & MCCORMICK, F. (2002) MDMX inhibits the p300/CBP-mediated acetylation of p53. *DNA Cell Biol,* 21, 519-25.

SCHMID, T., ZHOU, J. & BRUNE, B. (2004) HIF-1 and p53: communication of transcription factors under hypoxia. *J Cell Mol Med,* 8, 423-31.

SEJIMA, T. & MIYAGAWA, I. (1999) Expression of bcl-2, p53 oncoprotein, and proliferating cell nuclear antigen in renal cell carcinoma. *Eur Urol,* 35, 242-8.

SENGUPTA, S., LOHSE, C. M., CHEVILLE, J. C., LEIBOVICH, B. C., THOMPSON, R. H., WEBSTER, W. S., FRANK, I., ZINCKE, H., BLUTE, M. L. & KWON, E. D. (2006) The preoperative erythrocyte sedimentation rate is an independent prognostic factor in renal cell carcinoma. *Cancer,* 106, 304-12.

SHARP, D. A., KRATOWICZ, S. A., SANK, M. J. & GEORGE, D. L. (1999) Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J Biol Chem,* 274, 38189-96.

SHERR, C. J. (2000) The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res,* 60, 3689-95.

SHERR, C. J. (2006) Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer,* 6, 663-73.

SHERR, C. J. & WEBER, J. D. (2000) The ARF/p53 pathway. *Curr Opin Genet Dev,* 10, 94-9.

SHI, S. T., YANG, G. Y., WANG, L. D., XUE, Z., FENG, B., DING, W., XING, E. P. & YANG, C. S. (1999) Role of p53 gene mutations in human esophageal

carcinogenesis: results from immunohistochemical and mutation analyses of carcinomas and nearby non-cancerous lesions. *Carcinogenesis,* 20, 591-7.

SHIINA, H., IGAWA, M., URAKAMI, S., SHIRAKAWA, H., ISHIBE, T. & KAWANISHI, M. (1997) Clinical significance of immunohistochemically detectable p53 protein in renal cell carcinoma. *Eur Urol,* 31, 73-80.

SHINOZAKI, T., NOTA, A., TAYA, Y. & OKAMOTO, K. (2003) Functional role of Mdm2 phosphorylation by ATR in attenuation of p53 nuclear export. *Oncogene,* 22, 8870-80.

SHVARTS, A., BAZUINE, M., DEKKER, P., RAMOS, Y. F., STEEGENGA, W. T., MERCKX, G., VAN HAM, R. C., VAN DER HOUVEN VAN OORDT, W., VAN DER EB, A. J. & JOCHEMSEN, A. G. (1997) Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. *Genomics,* 43, 34-42.

SHVARTS, A., STEEGENGA, W. T., RITECO, N., VAN LAAR, T., DEKKER, P., BAZUINE, M., VAN HAM, R. C., VAN DER HOUVEN VAN OORDT, W., HATEBOER, G., VAN DER EB, A. J. & JOCHEMSEN, A. G. (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. *Embo J,* 15, 5349-57.

SHVARTS, O., SELIGSON, D., LAM, J., SHI, T., HORVATH, S., FIGLIN, R., BELLDEGRUN, A. & PANTUCK, A. J. (2005) p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. *J Urol,* 173, 725-8.

SIGAL, A. & ROTTER, V. (2000) Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res,* 60, 6788-93.

SIGALAS, I., CALVERT, A. H., ANDERSON, J. J., NEAL, D. E. & LUNEC, J. (1996) Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. *Nat Med,* 2, 912-7.

SINGH, A. D., SHIELDS, C. L. & SHIELDS, J. A. (2001) von Hippel-Lindau disease. *Surv Ophthalmol,* 46, 117-42.

SINIK, Z., ALKIBAY, T., ATAOGLU, O., BIRI, H., SOZEN, S., DENIZ, N., KARAOGLAN, U. & BOZKIRLI, I. (1997) Nuclear p53 overexpression in bladder, prostate, and renal carcinomas. *Int J Urol,* 4, 546-51.

SLUSS, H. K., ARMATA, H., GALLANT, J. & JONES, S. N. (2004) Phosphorylation of serine 18 regulates distinct p53 functions in mice. *Mol Cell Biol,* 24, 976-84.

SMIRNOV, M. N., SMIRNOV, V. N., BUDOWSKY, E. I., INGE-VECHTOMOV, S. G. & SEREBRJAKOV, N. G. (1967) Red pigment of adenine-deficient yeast Saccharomyces cerevisiae. *Biochem Biophys Res Commun,* 27, 299-304.

SOBKIEWICZ, A. & TWARDOWSKI, T. (1998) [Utilization of antisense oligonucleotide strategy in study of exposing and localization of rRNA on surface ribosomal subunits]. *Postepy Biochem,* 44, 55-63.

STAD, R., LITTLE, N. A., XIRODIMAS, D. P., FRENK, R., VAN DER EB, A. J., LANE, D. P., SAVILLE, M. K. & JOCHEMSEN, A. G. (2001) Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. *EMBO Rep,* 2, 1029-34.

STAD, R., RAMOS, Y. F., LITTLE, N., GRIVELL, S., ATTEMA, J., VAN DER EB, A. J. & JOCHEMSEN, A. G. (2000) Hdmx stabilizes Mdm2 and p53. *J Biol Chem,* 275, 28039-44.

STAMBOLIC, V., MACPHERSON, D., SAS, D., LIN, Y., SNOW, B., JANG, Y., BENCHIMOL, S. & MAK, T. W. (2001) Regulation of PTEN transcription by p53. *Mol Cell,* 8, 317-25.

STREIT, S., MICHALSKI, C. W., ERKAN, M., KLEEFF, J. & FRIESS, H. (2009) Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. *Nat Protoc,* 4, 37-43.

SUZUKI, Y., TAMURA, G., SATODATE, R. & FUJIOKA, T. (1992) Infrequent mutation of p53 gene in human renal cell carcinoma detected by polymerase chain reaction single-strand conformation polymorphism analysis. *Jpn J Cancer Res,* 83, 233-5.

TAN, M. H., ROGERS, C. G., COOPER, J. T., DITLEV, J. A., MAATMAN, T. J., YANG, X., FURGE, K. A. & TEH, B. T. (2004) Gene expression profiling of renal cell carcinoma. *Clin Cancer Res,* 10, 6315S-21S.

TAO, W. & LEVINE, A. J. (1999) P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. *Proc Natl Acad Sci U S A,* 96, 6937-41.

TOLEDO, F., LEE, C. J., KRUMMEL, K. A., RODEWALD, L. W., LIU, C. W. & WAHL, G. M. (2007) Mouse mutants reveal that putative protein interaction sites in the p53 proline-rich domain are dispensable for tumor suppression. *Mol Cell Biol,* 27, 1425-32.

TOLEDO, F. & WAHL, G. M. (2006) Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer,* 6, 909-23.

UCHIDA, C., MIWA, S., KITAGAWA, K., HATTORI, T., ISOBE, T., OTANI, S., ODA, T., SUGIMURA, H., KAMIJO, T., OOKAWA, K., YASUDA, H. & KITAGAWA, M. (2005) Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. *EMBO J,* 24, 160-9.

UCHIDA, T., GAO, J. P., WANG, C., JIANG, S. X., MURAMOTO, M., SATOH, T., MINEI, S., SHIMURA, S., IRIE, A., KAMEYA, T. & BABA, S. (2002) Clinical significance of p53, mdm2, and bcl-2 proteins in renal cell carcinoma. *Urology,* 59, 615-20.

UHLMAN, D. L., NGUYEN, P. L., MANIVEL, J. C., AEPPLI, D., RESNICK, J. M., FRALEY, E. E., ZHANG, G. & NIEHANS, G. A. (1994) Association of immunohistochemical staining for p53 with metastatic progression and poor survival in patients with renal cell carcinoma. *J Natl Cancer Inst,* 86, 1470-5.

UZUNLAR, A. K., SAHIN, H., YILMAZ, F. & OZEKINCI, S. (2005) Expression of p53 oncoprotein and bcl-2 in renal cell carcinoma. *Saudi Med J,* 26, 37-41.

VASAVADA, S. P., NOVICK, A. C. & WILLIAMS, B. R. (1998) P53, bcl-2, and Bax expression in renal cell carcinoma. *Urology,* 51, 1057-61.

VASSILEV, L. T., VU, B. T., GRAVES, B., CARVAJAL, D., PODLASKI, F., FILIPOVIC, Z., KONG, N., KAMMLOTT, U., LUKACS, C., KLEIN, C., FOTOUHI, N. & LIU, E. A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science,* 303, 844-8.

VLATKOVIC, N., GUERRERA, S., LI, Y., LINN, S., HAINES, D. S. & BOYD, M. T. (2000) MDM2 interacts with the C-terminus of the catalytic subunit of DNA polymerase epsilon. *Nucleic Acids Res,* 28, 3581-6.

VOGELSTEIN, B., LANE, D. & LEVINE, A. J. (2000) Surfing the p53 network. *Nature,* 408, 307-10.

VOUSDEN, K. H. (2000) p53: death star. *Cell,* 103, 691-4.

VOUSDEN, K. H. & LU, X. (2002) Live or let die: the cell's response to p53. *Nat Rev Cancer,* 2, 594-604.

WAGA, S., HANNON, G. J., BEACH, D. & STILLMAN, B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature,* 369, 574-8.

WANG, C. & CHEN, J. (2003) Phosphorylation and hsp90 binding mediate heat shock stabilization of p53. *J Biol Chem,* 278, 2066-71.

WANG, E. (2005) RNA amplification for successful gene profiling analysis. *J Transl Med,* 3, 28.

WANG, X., TAPLICK, J., GEVA, N. & OREN, M. (2004) Inhibition of p53 degradation by Mdm2 acetylation. *FEBS Lett,* 561, 195-201.

WARBURTON, H. E., BRADY, M., VLATKOVIC, N., LINEHAN, W. M., PARSONS, K. & BOYD, M. T. (2005) p53 regulation and function in renal cell carcinoma. *Cancer Res,* 65, 6498-503.

WARIDEL, F., ESTREICHER, A., BRON, L., FLAMAN, J. M., FONTOLLIET, C., MONNIER, P., FREBOURG, T. & IGGO, R. (1997) Field cancerisation and polyclonal p53 mutation in the upper aero-digestive tract. *Oncogene,* 14, 163-9.

WATSON, I. R., LI, B. K., ROCHE, O., BLANCH, A., OHH, M. & IRWIN, M. S. Chemotherapy induces NEDP1-mediated destabilization of MDM2. *Oncogene,* 29, 297-304.

WEBER, J. D., TAYLOR, L. J., ROUSSEL, M. F., SHERR, C. J. & BAR-SAGI, D. (1999) Nucleolar Arf sequesters Mdm2 and activates p53. *Nat Cell Biol,* 1, 20-6.

WEI, C. L., WU, Q., VEGA, V. B., CHIU, K. P., NG, P., ZHANG, T., SHAHAB, A., YONG, H. C., FU, Y., WENG, Z., LIU, J., ZHAO, X. D., CHEW, J. L., LEE, Y. L., KUZNETSOV, V. A., SUNG, W. K., MILLER, L. D., LIM, B., LIU, E. T., YU, Q., NG, H. H. & RUAN, Y. (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell,* 124, 207-19.

WESTRA, W. H., OFFERHAUS, G. J., GOODMAN, S. N., SLEBOS, R. J., POLAK, M., BAAS, I. O., RODENHUIS, S. & HRUBAN, R. H. (1993) Overexpression of the p53 tumor suppressor gene product in primary lung adenocarcinomas is associated with cigarette smoking. *Am J Surg Pathol,* 17, 213-20.

WILFINGER, W. W., MACKEY, K. & CHOMCZYNSKI, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques,* 22, 474-6, 478-81.

WILLIAMS, I., RICHARDSON, J., STARKEY, A. & STANSFIELD, I. (2004) Genome-wide prediction of stop codon readthrough during translation in the yeast Saccharomyces cerevisiae. *Nucleic Acids Res,* 32, 6605-16.

WU, X., BAYLE, J. H., OLSON, D. & LEVINE, A. J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev,* 7, 1126-32.

WYNFORD-THOMAS, D. (1996) p53: guardian of cellular senescence. *J Pathol,* 180, 118-21.

XIRODIMAS, D. P., SAVILLE, M. K., BOURDON, J. C., HAY, R. T. & LANE, D. P. (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell,* 118, 83-97.

XU, J., LIU, L. Z., DENG, X. F., TIMMONS, L., HERSPERGER, E., STEEG, P. S., VERON, M. & SHEARN, A. (1996) The enzymatic activity of Drosophila

AWD/NDP kinase is necessary but not sufficient for its biological function. *Dev Biol,* 177, 544-57.

YANG, J. C., HAWORTH, L., SHERRY, R. M., HWU, P., SCHWARTZENTRUBER, D. J., TOPALIAN, S. L., STEINBERG, S. M., CHEN, H. X. & ROSENBERG, S. A. (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med,* 349, 427-34.

YANG, J. Y., ZONG, C. S., XIA, W., WEI, Y., ALI-SEYED, M., LI, Z., BROGLIO, K., BERRY, D. A. & HUNG, M. C. (2006) MDM2 promotes cell motility and invasiveness by regulating E-cadherin degradation. *Mol Cell Biol,* 26, 7269-82.

YANG, Y., XIA, Q. & LIAN, F. (1999) [p53 mutations and protein overexpression in primary colorectal cancer and its liver metastasis]. *Zhonghua Zhong Liu Za Zhi,* 21, 114-8.

YOGOSAWA, S., MIYAUCHI, Y., HONDA, R., TANAKA, H. & YASUDA, H. (2003) Mammalian Numb is a target protein of Mdm2, ubiquitin ligase. *Biochem Biophys Res Commun,* 302, 869-72.

YU, J., WANG, Z., KINZLER, K. W., VOGELSTEIN, B. & ZHANG, L. (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci U S A,* 100, 1931-6.

ZHANG, X. H., TAKENAKA, I., SATO, C. & SAKAMOTO, H. (1997) p53 and HER-2 alterations in renal cell carcinoma. *Urology,* 50, 636-42.

ZHANG, Y., WOLF, G. W., BHAT, K., JIN, A., ALLIO, T., BURKHART, W. A. & XIONG, Y. (2003) Ribosomal protein L11 negatively regulates oncoprotein

MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol Cell Biol,* 23, 8902-12.

ZHANG, Y. & XIONG, Y. (1999) Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell,* 3, 579-91.

ZHANG, Z., WANG, H., LI, M., RAYBURN, E. R., AGRAWAL, S. & ZHANG, R. (2005) Stabilization of E2F1 protein by MDM2 through the E2F1 ubiquitination pathway. *Oncogene,* 24, 7238-47.

ZHAO, Y., CHAISWING, L., VELEZ, J. M., BATINIC-HABERLE, I., COLBURN, N. H., OBERLEY, T. D. & ST CLAIR, D. K. (2005) p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase. *Cancer Res,* 65, 3745-50.

ZHOU, B. P., LIAO, Y., XIA, W., ZOU, Y., SPOHN, B. & HUNG, M. C. (2001) HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol,* 3, 973-82.

ZIGEUNER, R., RATSCHEK, M., REHAK, P., SCHIPS, L. & LANGNER, C. (2004) Value of p53 as a prognostic marker in histologic subtypes of renal cell carcinoma: a systematic analysis of primary and metastatic tumor tissue. *Urology,* 63, 651-5.

7 Appendices

7.1 Appendix 1. Sequence analysis of p53 in RCC tumour samples

Sample	Mutation
1-1	Wt
1-2	Poor quality sequence
1-3	Wt
2-1	Wt
2-2	Wt
2-3	T633 deletion
3-1	Wt
3-2	Wt
3-3	C509T substitution
4-1	Wt
4-2	Wt
4-3	Wt
4-4	Wt
5-1	Wt
5-2	C785T mismatch
5-3	Wt
6-1	Wt
6-2	T853 deletion
6-3	G734A substitution
7-1	G733T substitution (G245C)
7-2	G733T substitution (G245C)
8-1	Wt
8-2	T852 deletion
8-3	Wt
9-1	T635 deletion
9-2	T605 deletion
9-3	Wt

10-1	C406G substitution (Q136E)
10-2	C406G substitution (Q136E)
11-1	Wt
11-2	C916T substitution
11-3	G725A substitution
12-1	C530T substitution
12-2	T821 deletion
12-3	G596A substitution
13-1	C497A substitution
13-2	Wt
13-3	G848A substitution
14-1	Wt
14-2	Wt
14-3	Wt
15-1	Wt
15-2	Wt
15-3	Wt

Table 7.1 Sequencing results of red yeast colonies obtained in FASAY. The left column shows a sample number (tumours 1-15 and two to four clones for each tumour sample) and identified mutations of p53 are shown in the right column. Mutations that were identified and confirmed by direct sequencing of at least two independent clones are shown in red.

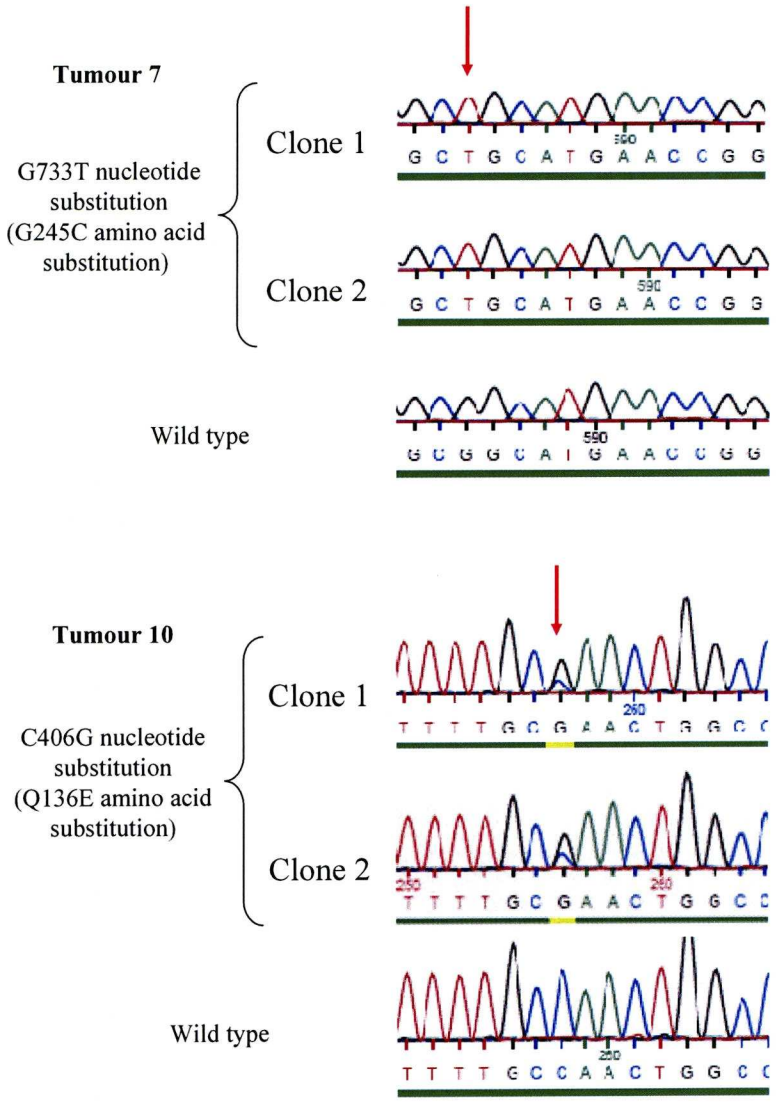

Figure 7.1 Mutations identified in tumour samples nr 7 and 10. The figure presents chromatograms from direct sequencing (performed by Eurofins company) of wt and mutated p53 from RCC tumour samples identified using FASAY.

7.2 Appendix 2. Abstracts of manuscripts submitted for publications that have arisen as a result of this study.

Manuscript 1

MDM2 and NME2 interact and have opposing effects on cell motility

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Abstract

Up-regulation of MDM2, combined with high levels of p53, are associated with poor overall survival in renal cell carcinoma. We therefore set out to identify MDM2 interacting proteins that could act as mediators of MDM2 oncogenic effects in renal cells. Using affinity chromatography and MS/MS we identified the non-metastatic cells 2, protein; NME2, a nucleoside diphosphate kinase, as an MDM2 interacting protein in HEK293 cell extracts. NME2 was also identified in parallel studies using a yeast two-hybrid screen for MDM2 interacting proteins expressed in renal cancer cells. The interaction is highly specific, as the closely related protein NME1 (87.5% amino acid identity) does not interact with MDM2 in yeast. Using measurements of p53 transcriptional activity as surrogates for MDM2 activity, we find that NME2 inhibits p53 activity in both normal and tumour cells. However, studies using Mdm2

-/- MEFs demonstrate that this occurs independently of MDM2. It is also independent of NME2 kinase activity. Specific NME proteins possess a well documented ability to suppress cell motility and metastasis and we show that NME2 suppresses motility under conditions where normal physiological levels of MDM2 are expressed. However, when higher levels of MDM2 are present, NME2 can no longer inhibit motility. This is not due to MDM2 promoting degradation of NME2 and is p53-independent. Our studies link MDM2 (and indirectly p53) with a key suppressor of motility and metastasis and may provide a mechanism to explain the association between MDM2 expression and poor patient survival.

Manuscript 2

MDM2 links poor survival and increased invasiveness in renal cell carcinoma

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Abstract

Purpose

Renal cell carcinomas (RCC) display an unusual pattern of co-upregulated p53 and MDM2 expression associated with disease progression and we find that the up-regulated p53 in RCC is frequently wild-type and is responsible for the co-up-regulation of MDM2. We also investigated the cellular mechanism by which p53/MDM2 up-regulation promotes disease progression and propose a novel consequence of MDM2 expression in RCC cells in promoting increased motility and invasiveness.

Patients and Methods

90 patients diagnosed with RCC were examined by IHC, with FASAY and genetic analysis of p53 IHC positive cases. Motility and invasion assays were performed on RCC cells with modulated p53/MDM2 levels.

Results

p53 mutations are rare amongst p53 positive RCCs. Up-regulation of wild-type p53 in RCC promotes MDM2 up-regulation *in vivo* (P=0.000012) and *in vitro* (P=0.01). Co-upregulation of p53 and MDM2 identifies patients with significantly reduced disease specific survival (DSS) by univariate (P=0.036) and Cox multiple regression analysis (P=0.027, RR=3.20). p53 dependent MDM2 up-regulation in RCC cells promotes increased motility and invasion.

Conclusions

p53/MDM2 expression identifies RCC patients with reduced DSS and we show for the first time that MDM2 is a critical regulator of probable determinants of this; cell motility and invasion in cells. The capacity of MDM2 to promote increased motility is independent of both p53 and of its intrinsic E3 ubiquitin ligase activity. Our studies thus identify MDM2 as an important subject for further investigation in RCC which likely acts via protein-protein interactions which potentially constitute a novel drug target.